PLANT ANIMAL INTERACTIONS

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Exogenous application of methyl jasmonate elicits defenses in Norway spruce (*Picea abies*) and reduces host colonization by the bark beetle *Ips typographus*

Abstract The terpenoid and phenolic constituents of conifers have been implicated in protecting trees from infestation by bark beetles and phytopathogenic fungi, but it has been difficult to prove these defensive roles under natural conditions. We used methyl jasmonate, a well-known inducer of plant defense responses, to manipulate the biochemistry and anatomy of mature *Picea abies* (Norway spruce) trees and to test their resistance to attack by *Ips typographus* (the spruce bark beetle). Bark sections of *P. abies* treated with methyl jasmonate had significantly less *I. typographus* colonization than bark sections in the controls and exhibited shorter parental galleries and fewer eggs had been deposited. The numbers of beetles that emerged and mean dry weight per beetle were also significantly lower in methyl jasmonate-treated bark. In addition, fewer beetles were attracted to conspecifics tunneling in methyl jasmonate-treated bark. Stem sections of *P. abies* treated with methyl jasmonate had an increased number of traumatic resin ducts and a higher concentration of terpenes than untreated sections, whereas the concentration of soluble phenolics did not differ between treatments. The increased amount of terpenoid resin present in methyl jasmonate-treated bark could be directly responsible for the observed decrease in *I. typographus* colonization and reproduction.

Keywords Conifers · Induced defenses · Phenolics · Terpenes

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Introduction

Bark beetles (Coleoptera: Scolytidae) feed, mate and oviposit in the subcortical tissues of host trees, and some species must attack and kill living trees in order to complete their life cycles. During the initial phase of host selection, beetles respond to visual (Strom et al. 1999) or chemical cues (Byers et al. 1988; Borden 1989), and enter or leave the host based on short-range chemical and tactile stimuli (Moeck et al. 1981; Raffa and Berryman 1983). If the host is acceptable, the beetles bore through the outer bark, excavate a nuptial chamber in the phloem and produce aggregation pheromones that attract conspecifics (Wood 1982). After mating, the female excavates egg galleries and deposits eggs along the gallery walls. The developing larvae feed on phloem and/or fungi in the larval galleries. The full development from egg to adult takes from one month to several years, depending on the species.

As the adult bark beetles enter the tree they introduce a variety of microorganisms, including phytopathogenic fungi. Conifers appear to have evolved sophisticated constitutive and inducible defense mechanisms against bark beetle colonization (reviewed by Franceschi et al. 2005). In the Pinaceae, terpenoid and phenolic compounds seem to play a central role in the constitutive defense system. These chemicals occur in phenolic parenchyma cells, resin ducts, resin blisters, and resin cells in the phloem and xylem (Bannan 1936; Hudgins et al. 2004) and provide immediate resistance to invasion. An invasion also activates inducible defenses, which may include secondary resin production (Raffa

and Berryman 1983; Christiansen 1985; Croteau et al. 1987; Alfaro 1995; Klepzig et al. 1995; Luchi et al. 2005) and production of additional phenolics, leading to qualitative and quantitative changes in chemical composition (Brignolas et al. 1995; Klepzig et al. 1995; Bonello and Blodgett 2003; Lieutier et al. 2003; Blodgett et al. 2005). Collectively, these constitutive and inducible compounds may deter beetle invasion, impede fungal growth, and seal entrance wounds (Berryman 1972; Klepzig et al. 1995; Phillips and Croteau 1999; Franceschi et al. 2005). However, to date, researchers have found it difficult to prove the exact role of host chemistry in bark beetle defense because of the difficulties involved with manipulating these biochemical pathways. We are thus exploring new ways to manipulate inducible defenses of conifers in a realistic experimental setting.

Jasmonates are endogenous plant phytohormones that are involved in defense signaling (Creelman and Mullet 1997). They have been intensively studied in the context of induced defense against herbivores (Creelman and Mullet 1997; Thaler et al. 2001). While most of this work has been done on angiosperms, there is growing evidence that jasmonates also induce defensive compounds in gymnosperms (Yukimune et al. 1996; Ketchum et al. 1999; Richard et al. 2000; Franceschi et al. 2002; Martin et al. 2002; Hudgins and Franceschi 2004). For example, methyl jasmonate (MJ) induces extensive biochemical and anatomical changes in Norway spruce, *Picea abies* (L.) Karst., similar to those caused by pathogens or artificial wounding (Franceschi et al. 2002; Martin et al. 2002, 2003). These putative defensive responses have recently been implicated in protection against the bark beetle-associated fungus, *Ceratocystis polonica* (Zeneli et al. 2006).

In the present investigation, we studied the host colonization process of the spruce bark beetle *Ips typographus* (L.) in mature Norway spruce trees treated with MJ. *I. typographus* is the most aggressive tree-killing bark beetle in Europe and has killed more than 50 million m^3 of Norway spruce in large outbreaks since the late 1940s (Worrell 1983; Christiansen and Bakke 1988). To test whether induced responses of *P. abies* to MJ also have the potential to defend against bark beetles, we applied MJ to mature trees in a forest stand and evaluated colonization of Norway spruce by *I. typographus* in the field and laboratory.

Materials and methods

Standing tree experiment

The objective of this experiment was to investigate the effects of MJ treatment on beetle colonization of live trees. In the spring of 2003, twelve trees were randomly selected in a naturally regenerated stand of mature Norway spruce in Ås, South East Norway [60 years old, tree height ∼28 m, diameter at breast height 27.85 ± 3.09 (SD) cm]. The population level of *I. typographus* in the area was low to moderate during the course of the experiments. On 26 May, a stem section between 1.5 and 4.5 m above ground was divided into east- and west-facing halves by two vertical lines, using a water-based latex paint. One half of each tree was treated with 100 mM MJ and the other half was left untreated to serve as a control (MJ_C). MJ was sprayed onto the stem using a small spray gun, while carefully avoiding contamination of the control side.

Three weeks after MJ application, four samples containing the bark and outermost sapwood (1.6 cm wide × 5 cm high × 1 cm deep) were removed for anatomical investigation from each tree at 1.5 and 3.5 m above ground, two on the treated side and two on the control side. At each site a smaller sample (1.6×1.6×1 cm) for analyzing terpenes and phenolics was removed, quickly frozen on liquid N_2 and transferred to a −80 °C freezer. The anatomical samples were immediately placed in fixative (2% paraformaldehyde and 1.25% glutaraldehyde in 50 mM L-piperazine-N-N'-bis(2-ethane sulfonic) acid buffer, pH 7.2).

On 17 June, an Ipslure pheromone dispenser (Borregaard, Sarpsborg, Norway) was placed on each tree 2 m above ground to induce attack by *I. typographus*. The dispensers were placed on the north side of the trees on the border between the MJ-treated and untreated sides. Because the beetle population in the area was relatively low and the main flight of the beetles had already taken place, an additional Ipslure dispenser was added 3 days later to enhance attraction. Beetle aggregation remained moderate on all trees except one, which was located on the south-facing edge of the stand. This tree was mass-attacked and killed by the beetles, as opposed to the other 11 trees, which survived with moderate attacks. The pheromone dispensers remained on the stems until 25 July, when the trees were sampled to assess beetle success.

To assess beetle attacks, the outer cork bark was carefully shaved away on both sides of trees at the dispenser height. A transparent plastic sheet (210×297 mm) was placed on the stem within the shaved area, with the long side oriented vertically, and well away from the dividing lines between the two treatments. All entrance holes covered by the sheet and penetrating into the live phloem were marked, and the more developed beetle galleries were traced. On 26 August, the trees were sampled again immediately above the first sampling site, using the same method. The tree that had been mass-attacked and killed was excluded from the experiment, since the entire bark was crowded with well-developed beetle galleries. In the laboratory we recorded the number of entrance holes and incipient galleries (tunnels > 10 mm length), and the total length of all maternal galleries on the plastic sheets. When multiple galleries extended from a single entrance hole, we recorded the sum of their lengths.

On 24 July, 2003, 12 other Norway spruce trees in the same stand (DBH = 28.59 ± 3.19 cm) were treated with MJ as described above to see if MJ treatment in one year

would have any effects on beetle colonization the following year. Samples for anatomical and chemical analyses were removed from the trees the following spring (12 May 2004) as described above, and two days later the trees were baited with pheromone dispensers to induce attack by *I. typographus*. At this stage, there was extensive resin flow in some trees on bark that had been treated with MJ. On 16–17 June 2004, the outer bark was removed and the outcome of the beetle attacks was assessed as described above.

In both years, all anatomical samples were taken to the laboratory and processed as described in Krokene et al. (2003). Briefly, samples were rinsed with buffer, dehydrated in a graded series of ethanol, and embedded in acrylic resin. Cross-sections (1 μm thick) were cut on a diamond knife, dried onto gelatin-coated slides, stained with Stevenel's blue (Del Cerro et al. 1980), and mounted with immersion oil. Digital images were recorded at a magnification of 5× using a Leica (Solms, Germany) DC300 CCD camera mounted on a Leitz (Oberkochen, Germany) Aristoplan photomicroscope. The extent of traumatic resin duct (TD) formation in the xylem was quantified as the percentage of tracheid lanes that contained TDs (including the epithelial cells lining the ducts) across the full tangential width of each section (1,380 μm).

Petri dish assay

The objective of this experiment was to investigate the effects of MJ treatment on beetle colonization, egg laying, and attraction of conspecifics. The population level of *I. typographus* in the area was low to moderate during the course of the experiments. On 7 May 2004, a 3 m section of the lower stem of a 40-year-old clonal Norway spruce tree growing in Ås, South East Norway was treated in the same way as in the standing tree experiment, with half the bark circumference treated with 100 mM MJ and the other half left untreated. Three and a half weeks later the tree was felled and the 3 m stem section was cut into four bolts. An untreated ramet of the same clone was felled and sectioned the same way. The bolts were sealed at both ends with melted paraffin wax to minimize desiccation, and stored at 4 °C for later use.

Assay units consisted of fresh bark-phloem disks in large Petri dishes (150 mm in diameter, 25 mm deep), as described by Erbilgin and Raffa (2000). Briefly, phloem and outer bark were peeled off the stored bolts and cut into 150 mm-diameter disks (177 cm²) using a hole saw. The disks were then placed with the cambium facing downwards in Petri dishes with melted paraffin wax applied to the bottom and edges of the disks to minimize desiccation of the phloem. Three treatments were tested: (1) bark from the treated half of the MJ-treated tree (MJ), (2) bark from the untreated half of the same tree (MJ$_C$), and (3) bark from the untreated ramet (control). Bark beetles were collected in traps baited with Ipslure

pheromone dispensers and kept at 60–65% RH and 4 °C for up to ten days until use. The beetles used in the assays were not sexed beforehand, because male and female *I. typographus* cannot be reliably distinguished based on external morphology. Beetles were sexed after the experiment by dissecting their genitals under the microscope. However, traps with synthetic pheromones are known to capture roughly equal amounts of each sex, and to ensure that both sexes would be present in each assay unit, we introduced ten beetles per unit. The Petri dishes were covered by a mesh screen to prevent the beetles from escaping.

The beetles were allowed to colonize the bark disks for 24 h at 23 °C before the units were suspended from flight interception traps in the field. The traps consisted of two thin sheets of clear acrylic plastic (39 cm high × 21 cm wide) mounted vertically and crosswise over a plastic funnel (22 cm diameter) fitted with a collecting bottle. One assay unit was placed as bait within the funnel, just below the plastic sheets. Fifteen traps were deployed in openings within a spruce stand, constituting five blocks in a randomized complete block design, with 15 m between treatments within a block and 50 m between blocks. The traps were placed with the collecting bottle hanging close to the top of the ground vegetation. The assay units were replaced with fresh units (prepared from the cold stored bolts) and re-randomized every five days over a period of 15 days (3–17 June, 2004), providing a total of 15 replicates per treatment.

After each five-day trapping period, the bark disk was dissected immediately, and the number of male entrance holes, the number of maternal galleries, the total length of maternal galleries and the total number of egg niches were recorded per disk. Only beetles that had tunneled more than 10 mm were considered to be successful colonizers. Insects caught in the traps were identified to species and sexed after dissection under the microscope.

Log experiment

Another experiment was conducted to investigate effects of MJ treatment on brood production of *I. typographus*. We utilized two ramets of a different 40-year-old clone growing in the same stand that was used for the Petri dish experiment. The trees were treated in the same way as in the Petri dish assay, and on 15 June 2004 the trees were felled and cut into 40 cm-long bolts. This yielded eight bolts where half the bark circumference was treated with MJ (MJ) and the other half was untreated (MJ$_C$), and eight bolts where the whole bark area was untreated (control). Pairs of bolts (one from each type) were placed vertically on the forest floor in a randomized block design and baited with an Ipslure pheromone dispenser placed between the bolts. The distance between bolts within a pair was 0.5 m and the distance between pairs was 50 m. Bolts were left in the field for

seven days for colonization by *I. typographus* and then brought to an outdoor insectary. The bolts were split longitudinally through the center to separate the MJ- and MJ$_C$-treatments, and each half-bolt was sealed with melted paraffin wax on the cut surfaces to minimize desiccation. Control bolts were split and treated in the same way as the MJ-treated bolts. This procedure yielded 32 half-bolts that were hung individually inside fine mesh cloth bags with funnels and collection bottles attached to the lower end, to collect emerging beetles.

In October–November 2004, we counted the number of beetles that had emerged from each half-bolt. Because there still were beetles in the bolts, we also estimated the number of remaining beetles by counting the numbers under two randomly chosen 100 cm^2 (10×10 cm^2) areas of bark on each bolt. Furthermore, we determined beetle dry weight by weighing 100 randomly selected beetles per bolt (dried at 110 °C for 60 min). Beetles were weighed to an accuracy of 0.01 mg.

Chemical analyses

Terpene extractions were based on the procedures of Zeneli et al. (2006). Briefly, wood and bark tissue (100 mg each) were submerged separately in 1.5 ml of tert-butyl methyl ether containing 150 μg ml^{-1} isobutylbenzene and 200 μg ml^{-1} dichlorodehydroabietic acid as internal standards. The tissue samples were extracted over 14 h with constant shaking at room temperature. The ether extract was transferred to a fresh vial and washed with 0.3 ml of 0.1 M (NH$_4$)$_2$CO$_3$ (pH 8.0) to purify extracted terpenes from other small organic acids. The extract was then split into two equal portions. One portion of the extract was prepared for monoterpene and sesquiterpene analysis by filtering through a Pasteur pipette column filled with 0.3 g of silica gel (Sigma 60 Å) overlaid with 0.2 g of anhydrous MgSO$_4$. The column was washed with 1 ml of diethyl ether, and the eluent was collected in a fresh vial. Finally, the sample was concentrated to an approximate volume of 100 μl that was stored at −20 °C until analysis. In the second portion of the extract, the diterpene resin acids were methylated by adding 50 μl of 0.2 M *N*-trimethylsulfonium hydroxide in methanol (Macherey-Nagel GmbH & Co, Germany) to 0.4 ml of the washed ether extract in a separate vial and incubated at room temperature for 2 h to allow the methylation reaction to go to completion. The solvent was then evaporated under nitrogen to about 100 μl, leaving the residual methyl-esterified diterpene fraction for analysis via GC-MS.

A Hewlett–Packard (Palo Alto, CA, USA) 6890 GC-MSD system, using a DB-5 MS column (30 m × 0.25 mm × 0.25 μm, J&W Scientific, Folsom, CA, USA), was used for the GC-MS analysis of monoterpenes and sesquiterpenes. Split injections (1 μl ether extract) were made at a ratio of 1:5 for wood and 1:10 for bark samples with an injector temperature of 220 °C. The instrument was run under the same program

described by Zeneli et al. (2006). Identification of terpenes was based on comparison of retention times and mass spectra with authentic standards or with mass spectra in the Wiley or National Institute of Standards and Technology libraries.

Analysis of diterpene constituents was performed on the same GC-MS instrument fitted with the same DB-5 MS column. Injections were made with 1 μl of the concentrated, derivatized ether extracts. GC-MS split ratios were 1:10 (for both wood and bark extracts) with an injector temperature of 220 °C. The temperature programs for the instrument are described by Zeneli et al. (2006). GC-MS generated peaks were quantified using Hewlett–Packard Chemstation software. For quantitative analysis of monoterpenes, sesquiterpenes and diterpenes, the MS detector was operated in the SIM mode. The selected ions for the internal standards, monoterpenes, sesquiterpenes and diterpene methyl esters are described by Zeneli et al. (2006). The total monoterpene, sesquiterpene or diterpene resin acid content was calculated as the sum of the individually quantified compounds.

Extraction of phenolic constituents was modified from Laitinen et al. (2002) as described by Zeneli et al. (2006). Briefly, wood and bark tissues were weighed (100 mg) and extracted separately with 2.5 ml of methanol (100%) using an Ultra-Turrax (IKA-WERKE, Staufen, Germany) homogenizer for 30 s, after which the sample was left for 15 min on ice. The sample was then centrifuged (16,000*g*, 3 min) and decanted. The pellet was extracted three more times by homogenization for 30 s, placed for 2 min on ice, and then centrifuged. The combined supernatants were vacuum-evaporated to dryness, redissolved in 1 ml of 100% methanol, and transferred to a fresh vial for analysis. After being evaporated to dryness again under nitrogen, they were stored at −20 °C.

The quantitative analysis of phenolics of wood and bark samples was carried out by high-performance liquid chromatography (HPLC) using a Hewlett–Packard system with a quaternary pump (HP 1050), an autosampler (HP 1100 at 230 nm), a photodiode array detector (HP 1100), HP ChemStation Software, and a 5 μm Lunal C18 column (250×4.6 mm^2 ID) (Phenomenex, Torrance, CA, USA). The mobile phases used were 0.2% trifluoroacetic acid and acetonitrile. The gradients for separation and identification of the compounds are described by Zeneli et al. (2006).

Data analyses

Data were analyzed using analysis of variance. Each variable was tested to check that it satisfied assumptions of normality and homogeneity of variances (Zar 1996) by graphical analysis of residuals (Neter et al. 1983). If the variance was nonhomogeneous, variables were transformed to square root, which provided distributions that satisfied these assumptions in all

cases. Sex ratio and proportional data for *I. typographus* were transformed by arcsin$\sqrt{y}$. Beetle colonization data from the standing tree experiment were analyzed on a single-tree basis, by using the calculated differences between MJ-treated and untreated bark within trees as the response variable. The data were subjected to one-sample *t*-tests using SYSTAT (SPSS Inc., Chicago, IL, USA). Dependent variables in the Petri dish assay and log experiment were analyzed by repeated measure analysis in Proc Mixed (Littell et al. 1996), as a split plot, with randomized block design, treating sites, bark discs, or logs as blocks. For each variable, covariance parameter estimates (REML) for block and block by treatment were calculated in order to reveal the extent of variability due to block or block by treatment interaction. In all experiments, blocks were accepted as a random factor; if the covariance parameter of a block was zero, the block term was eliminated from the random statement in the model. Tukey's Protected LSD test was used for multiple comparisons of means.

Results

### Methyl jasmonate reduced *I. typographus* colonization of *P. abies* bark

In the standing tree experiments, no tunnels extended more than 50 mm from the entrance hole in the 11 surviving trees and no oviposition took place; this allowed comparison of the number and length of attacks between MJ-treated and untreated bark without considering attack success.

In 2003, MJ-treated bark (MJ) sustained significantly less bark beetle colonization than untreated bark on the same tree (MJ$_C$), at both the lower and upper sampling positions (Fig. 1; $P = 0.0001–0.05$). Treated bark had an average of 31% fewer entrance holes, 69% fewer galleries, and gallery length was 82% shorter than in untreated bark. All of these differences were statistically significant (Fig. 1). The difference between MJ$_C$ and MJ-treated bark was significantly greater for total gallery length (MJ$_C$:MJ ratio of 6.53) than for number of incipient galleries and entrance holes (MJ$_C$:MJ ratios of 2.40 and 1.66, respectively ($F = 4.48$, $P = 0.015$). This suggests that the negative impact of MJ increased as the beetles proceeded through the colonization sequence from first entry into the bark to sustained tunneling activity. Beetle colonization also varied significantly with sampling position, with more colonization on the lower position, which was closer to the pheromone source ($F = 4.24–13.75$, $P = 0.0006–0.04$ for the different colonization variables). These lower samples were probably more influenced by the proximity to the pheromone dispenser itself, and thus the upper samples should give a more unbiased view of beetle attack behavior. The effect of MJ treatment (measured as the difference between MJ$_C$ and MJ-treated bark within each tree) did not differ between sampling positions ($F = 0.01–2.06$, $P = 0.17–0.94$ for the different colonization variables).

In 2004, one year after MJ treatment, *I. typographus* attacks were induced on 12 previously unattacked trees. Although the number of beetle attacks in 2004 was much lower than in 2003 (115 vs. 434 attacks/m^2, respectively; $F = 82.51$, $P < 0.0001$), the attacks were much more successful in terms of total gallery construction (4.63 vs. 1.47 m tunnel/m^2; $F = 2.78$, $P = 0.10$) (Fig. 1). MJ treatment had no significant effect on beetle colonization in 2004 when the whole data set was analyzed ($P = 0.16–0.40$ for the different variables; one-sample *t*-test). However, if one tree with extensive beetle colonization on the MJ-treated half of the tree was excluded from the analysis, MJ-treated bark once again had significantly fewer galleries (56.85 vs. 82.35 per m^2, $P = 0.021$) and less total gallery length (3.51 vs. 5.82 m per m^2, $P = 0.048$) than control bark. At any rate, these differences were much smaller than those observed between MJ-treated and MJ$_C$ bark after the 2003 attacks (galleries: 41.54 vs. 117.33 per m^2; total gallery length: 0.58 vs. 2.37 m per m^2).

Fig. 1 Effects of methyl jasmonate (*MJ*) treatment of Norway spruce on *Ips typographus* colonization during the year of MJ treatment (2003) and the following year (2004) at two different heights above the ground. MJ treatment *white bars*, untreated controls *black bars*. Entries = number of beetles entering the bark, galleries = number of initiated egg galleries, length = total gallery length. *Bars* 1 SE, $n = 11$ in 2003 and 12 in 2004
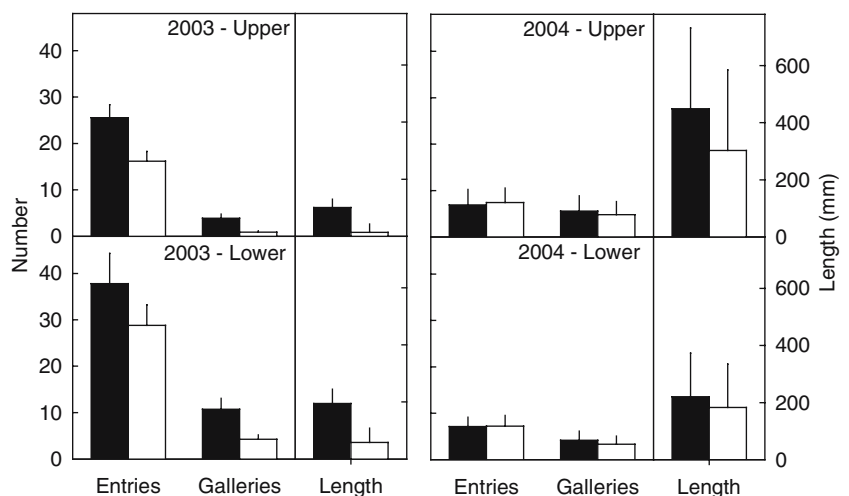
In the Petri dish assays, the number of beetle entrance holes in fresh bark disks did not vary among treatments, suggesting that MJ application did not affect the entry behavior of *I. typographus* (Table 1). However, in these assays MJ had significant effects on beetle behavior within the bark. The number of parental galleries, length of galleries, and number of eggs were all significantly higher in MJ-free than in MJ-treated bark (Table 1). There were no significant differences between disks from untreated parts of MJ-treated trees and those from completely untreated trees. The male:female ratio in each assay unit was similar among treatments [$F_{(2,24)} = 1.09$, $P = 0.367$ sex by treatment interaction], and ranged from 0.47 to 0.55.

Methyl jasmonate reduced *I. typographus* aggregation

Attraction of *I. typographus* by conspecifics that had colonized MJ-treated or untreated bark was tested by attaching Petri dishes with boring beetles to flight interception traps. A total of 273 bark beetle specimens were captured in these traps across treatments, including 170 *I. typographus* (89 female, 81 male), 25 *Hylastes cunicularius*, 43 *Dryocoetes autographus*, and 35 *Pityogenes chalcographus*. Use of MJ-treated bark disks significantly reduced the number of beetles attracted, as male *I. typographus* excavating in MJ-treated bark attracted fewer conspecifics than males excavating in MJ$_C$ bark or control bark (Table 2). There was also a significant effect of time [$F_{(2,24)} = 30.97$, $P < 0.0001$] and time by treatment interaction [$F_{(4,24)} = 3.23$, $P = 0.03$] on the number of beetles collected. This was probably due to variable weather conditions with frequent rainfall during parts of the trapping period. There was a significant correlation between the number of beetles attracted and mean gallery length in the bark discs ($P = 0.005$, $R^2 = 0.67$). The proportion of male vs. female *I. typographus* beetles attracted to the traps (averaging 47% male) was not affected by MJ treatment [$F_{(1,24)} = 0.79$, $P = 0.383$ for sex and $F_{(2,24)} = 0.98$, $P = 0.39$ for sex by treatment interaction]. *Pityogenes chalcographus* showed a similar response pattern as *I. typographus* (Table 2). For *H. cunicularius* and *D. autographus*, trap catches did not appear to differ among treatments. There was no time or time by treatment effects on any of the latter three species.

Methyl jasmonate reduced *I. typographus* reproduction in detached logs

In another experiment, bolts cut from untreated trees or trees that had been treated with MJ were placed along the edge of a Norway spruce forest and left to be colonized by *I. typographus* attracted by pheromone dispensers. Beetle reproduction was negatively affected by MJ treatment. Significant reductions occurred in total numbers of beetles produced per square diameter bark surface and mean dry weight per beetle when beetles colonized bolts cut from MJ-treated sections (Table 3, one pair of logs with results that were far outside the range of the other log pairs was omitted from the analysis).

Methyl jasmonate increased the number of traumatic resin ducts and the accumulation of terpene resin constituents

Anatomical analyses showed that in both years there were significantly more traumatic resin ducts (TDs) in the xylem of MJ-treated sections of trees than in the xylem of untreated control sections (2003: 27.7 vs. 1.8% of sapwood circumference, $P = 0.001$; 2004: 14.2 vs. 5.9%, $P = 0.04$; one-sample *t*-test). There was no significant difference in TD abundance in MJ-treated sections between years ($P = 0.11$, *t*-test).

The concentrations of monoterpenes, diterpenes and total terpenes were significantly higher in MJ-treated bark and sapwood than in control tissues (Table 4). This was true both for the year of MJ application (2003) and the following year, but the response was much weaker in 2004, particularly in the bark. Total terpene concentration in bark was 2.5-fold higher after MJ treatment in 2003 than in untreated portions of trees, but only 1.3-fold higher in 2004. In wood, the corresponding fold-differences were 3.0 and 2.1. There were no qualitative differences in terpene composition in MJ$_C$ versus MJ-treated bark or wood ($R^2 > 0.99$ for linear regression of

Table 1 Bark beetle colonization of Norway spruce bark treated with methyl jasmonate (MJ) and untreated bark (Control = bark from untreated trees; MJ$_C$ = bark from untreated parts of MJ-treated trees)

	No. entrance holes[a]	No. galleries	Gallery length (cm)	No. egg niches
Control	5.47 ± 0.26	3.60 ± 0.35 a	19.83 ± 2.86 a	36.67 ± 8.65 a
MJ$_C$	5.13 ± 0.32	4.07 ± 0.48 a	22.30 ± 2.25 a	45.53 ± 6.30 a
MJ	5.53 ± 0.24	2.47 ± 0.31 b	9.33 ± 1.29 b	10.80 ± 3.10 b
$F_{(2,12)}$	0.71	5.55	17.48	14.31
P	0.5125	0.0196	< 0.0001	0.0007

Means followed by the same letter in a column are not significantly different at $P < 0.05$, based on Proc Mixed and Tukey's Protected LSD test on transformed data ($\sqrt{y}$). Untransformed means ± SE are reported
[a]The mean number of males inside each assay unit was similar across treatments: 3.4 ± 0.7 for Control, 3.2 ± 0.9 for MJ$_C$, and 3.5 ± 1.0 for MJ. Sex ratio of beetles inside the assay unit was determined by dissection of insects at the end of the experiment

Table 2 Response of flying bark beetles to male and female *Ips typographus* tunneling in Norway spruce bark treated with methyl jasmonate (MJ) and untreated bark (Control = bark from untreated trees; MJ_C = bark from untreated parts of MJ-treated trees)

	Number of insects of each species trapped			
	I. typographus	*P. chalcographus*	*D. autographus*	*H. cunicularius*
Control	5.00 ± 0.82 a	1.33 ± 0.44 a	1.20 ± 0.29	0.73 ± 0.18
MJ_C	5.00 ± 1.08 a	0.87 ± 0.38 a	0.93 ± 0.43	0.60 ± 0.27
MJ	1.33 ± 0.33 b	0.13 ± 0.09 b	0.73 ± 0.27	0.33 ± 0.19
$F_{(2,12)}$	13.72	4.09	0.33	0.86
P	0.0008	0.044	0.723	0.448

Means (number of insects caught per trap and five-day collection period) followed by the same letter in a column are not significantly different at $P < 0.05$, based on Proc Mixed and Tukey's Protected LSD test on transformed data ($\sqrt{y}$). Untransformed means $\pm$ SE are reported

Table 3 Mean number of *Ips typographus* that emerged per dm^2 of bark surface, mean dry weight (dw) per beetle, and total beetle dw per dm^2 of bark surface on logs treated with methyl jasmonate (MJ), untreated logs (Control) and untreated halves of MJ-treated logs (MJ_C)

	No. beetles/dm^2	Mean dw/beetle (mg)	Total beetle dw/dm^2
Control	43.61 ± 3.37 a	6.17 ± 0.13 a	267.66 ± 19.86 a
MJ_C	42.36 ± 3.02 a	5.87 ± 0.18 a	249.08 ± 19.85 a
MJ	35.11 ± 2.71 b	5.17 ± 0.12 b	182.98 ± 17.62 b
$F_{(2,20)}$	5.54	13.1	6.53
P	0.012	0.0002	0.006

Means followed by the same letter in a column are not significantly different at $P < 0.05$, based on Proc Mixed and Tukey's Protected LSD test on transformed data ($\sqrt{y}$). Untransformed means $\pm$ SE are reported

percent composition of individual terpenes in MJ_C and MJ-treated tissues). A total of 27 different terpenes were detected with the monoterpenes α- and β-pinene and limonene, the sesquiterpene germacrene D, and the diterpenes dehydroabietic acid, isopimaric acid and neoabietic acid, making up nearly three-quarters of the total terpenes. The total amount of terpenes was roughly the same in the bark and wood (Table 4).

Much more soluble phenolics were in the bark than in the wood, both in terms of the number of individual compounds detected (23 vs. 9) and total quantities (Table 4). The stilbene glycosides astringin and isorhapontin dominated, making up about 67% of the total soluble phenolics in the bark and 50% in the wood. However, there were no quantitative differences in phenolic content between MJ-treated bark or sapwood and untreated tissue on the same tree (MJ_C), neither for individual compounds ($P > 0.22$, one-sample *t*-test) nor for total soluble phenolics (Table 4). Nor were there any qualitative differences in soluble phenolic composition between MJ-treated and untreated tissues ($R^2 = 0.85$ for wood and 0.99 for bark, linear regression of percent composition of individual phenolics in MJ_C and MJ-treated tissues).

Discussion

This study demonstrates that MJ application to mature *P. abies* induces resistance against bark beetle colonization. Bark sections of *P. abies* treated with MJ had significantly less *I. typographus* colonization than control bark, with shorter parental galleries and fewer eggs.

Table 4 Effect of methyl jasmonate (MJ) on terpene and phenolic content (mg g^{-1}) of Norway spruce trees during the year of treatment (2003) and the following year (2004)

	2003			2004		
	Control	MJ	P	Control	MJ	P
Bark						
Total monoterpenes	0.94 ± 0.07	2.33 ± 0.23	< 0.001	1.22 ± 0.15	1.48 ± 0.14	0.037
Total sesquiterpenes	0.19 ± 0.02	0.49 ± 0.09	0.085	0.21 ± 0.04	0.19 ± 0.04	1.000
Total diterpenes	0.82 ± 0.02	2.02 ± 0.05	< 0.001	1.11 ± 0.32	1.67 ± 0.48	0.012
Total terpenes	1.96 ± 0.10	4.84 ± 0.31	< 0.001	2.54 ± 0.27	3.35 ± 0.36	0.016
Total soluble phenolics	33.98 ± 3.0	29.40 ± 2.14	0.780	23.48 ± 6.78	26.29 ± 7.59	0.540
Wood						
Total monoterpenes	0.83 ± 0.06	2.42 ± 0.26	< 0.001	1.13 ± 0.11	2.22 ± 0.25	< 0.001
Total sesquiterpenes	0.11 ± 0.02	0.48 ± 0.11	0.014	0.17 ± 0.04	0.29 ± 0.06	0.042
Total diterpenes	0.76 ± 0.23	2.13 ± 0.64	< 0.001	0.92 ± 0.27	2.06 ± 0.59	< 0.001
Total terpenes	1.71 ± 0.13	5.04 ± 0.47	< 0.001	2.21 ± 0.13	4.56 ± 0.43	< 0.001
Total soluble phenolics	0.61 ± 0.18	0.50 ± 0.15	0.800	0.28 ± 0.08	0.32 ± 0.09	0.240

Treatments were compared using one-sample *t*-test ($n = 11$ in 2003, 12 in 2004). Untransformed means $\pm$ SE are reported

The number of beetles produced and mean dry weight per beetle were also significantly lower in MJ-treated bark. Furthermore, fewer beetles were attracted to conspecifics tunneling in MJ-treated bark.

These effects are discussed in relation to the host colonization sequence of bark beetles, from host selection to concentration, establishment and dispersal (Wood 1982), as shown diagrammatically in Fig. 2. The selection phase begins when the beetles respond to host stimuli before or after landing on the bark, and ends with sustained feeding in the phloem. MJ treatment did not appear to have strong effects on the beetle's decision to enter the bark (cf. the first two experiments), and hence any influence of MJ on host selection before or shortly after landing on the host is also likely to be small (Phase 1). However, the absence of clear effects of MJ on beetle entry could be due to our use of synthetic aggregation pheromones to initiate attacks on the trees. The pheromone emission from the dispensers corresponds to about 200 pheromone-producing males (Birgersson and Bergström 1989), and this signal might override any deterring effects of MJ treatment and so increase the propensity of the beetles to enter unsuitable hosts (Borden 1982; Camacho et al. 1994). However, since MJ did not affect entry behavior in the bark disc assay where no pheromones were used, this possibility seems unlikely. We did not test for an effect of MJ on primary

(host-induced) attraction of "pioneer beetles" (the first beetles to arrive on a tree). However, the total emission of volatile terpenes has been shown to increase fivefold after foliar application of MJ on two-year-old saplings, with individual compounds such as (E)-β-farnesene and linalool increasing up to 100-fold (Martin et al. 2003). These compounds are of particular ecological interest, as they have been reported to attract natural enemies of herbivores or repel herbivores directly in other plant species (Kessler and Baldwin 2001; Pichersky and Gershenzon 2002). The possibility that similar effects occur in the Norway spruce-*I. typographus* system deserves further investigation.

The concentration phase begins when flying beetles respond to aggregation pheromones and/or host compounds (secondary attraction), continues with the production of more aggregation pheromones, and ends with the production of antiaggregation pheromones (Phase 2/ Fig. 2). This process is crucial to the survival of the dispersing population of tree-killing bark beetles, since they rely on mass attacks of host trees to obtain substrate for larval development. Fewer *I. typographus* were attracted to beetles tunneling in MJ-treated bark as compared to beetles tunneling in untreated bark. Shorter gallery length in MJ-treated bark might be a reflection of differences in host quality and thus beetle attraction, since beetle attraction seems to be proportional to the extent of beetle tunneling. Similar results have been reported for the congeneric species *I. pini* Say (Erbilgin and Raffa 2000). Interference of plant defenses induced by MJ on pheromone emission could also explain the lower attraction of beetles in the current study. For example, Raffa and Berryman (1983) found that *Dendroctonus ponderosae* Hopkins beetles that were continuously fighting resin defenses in vigorous trees were unable to initiate mass attacks. Additional studies are necessary to understand how MJ interferes with pheromone production and communication in *I. typographus*.

Methyl jasmonate strongly inhibited the establishment phase of host colonization, which begins when the mortality of the host trees is assured (Phase 3/Fig. 2). This phase starts with the initiation of mating, gallery construction and oviposition and ends when elongation of egg galleries and oviposition cease. MJ treatment strongly influenced gallery construction and egg laying by *I. typographus*. Even though the total number of attacks was similar on both the treated and untreated sides of the trees, incipient gallery construction was much less extensive on the MJ-treated side, indicating that these tissues were less suitable for the beetles. One plausible explanation is that chemical changes in the phloem affected the ability or propensity of bark beetles to excavate host tissues and lay eggs. We do not currently know whether *I. typographus* is negatively affected by increased quantities of terpenes, but studies on *I. pini* indicate that tunneling in a phloem-based medium decreases with increasing concentration of α-pinene in the medium (Wallin and Raffa 2000). The phytopathogenic fungal associates of the beetles play a crucial role during


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┌─────────────────────────────┐
│ Hypothetical Effects of Methyl│
│   Jasmonate Application to   │
│     Mature Norway Spruce     │
└─────────────────────────────┘
              │
              ▼
┌──────────────────┐      ┌─────────────────────────────────┐
│    Phase 1:      │─────▶│ Before Landing: Host Selection: ? │
│  Host Selection  │      │ After Landing: Host Selection: ?  │
└──────────────────┘      │ Entering: NO?                     │
              │           └─────────────────────────────────┘
              ▼
┌──────────────────┐      ┌─────────────────────────────────┐
│    Phase 2:      │─────▶│ Attraction: YES                   │
│ Host Colonization│      └─────────────────────────────────┘
└──────────────────┘
              │
              ▼
┌──────────────────┐      ┌─────────────────────────────────┐
│    Phase 3:      │─────▶│ Gallery Construction: YES         │
│ Host Establishment│     │ Egg Laying: YES                   │
└──────────────────┘      │ Brood Production/Survival: YES    │
              │           └─────────────────────────────────┘
              ▼
┌──────────────────┐      ┌─────────────────────────────────┐
│    Phase 4:      │─────▶│ Emergence: YES                    │
│    Dispersal     │      │ Dispersal: ?                      │
└──────────────────┘      └─────────────────────────────────┘
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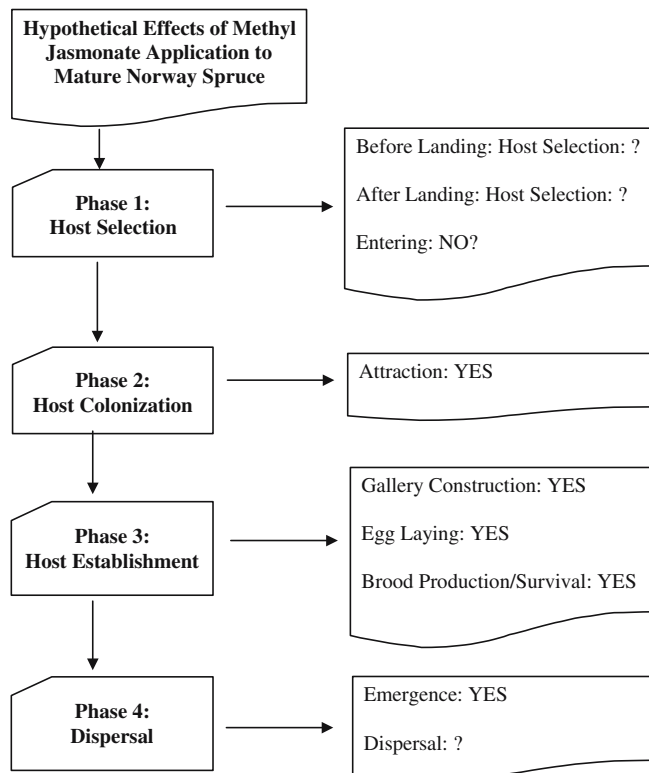
Fig. 2 Conceptual diagram of the different stages in *I. typographus* host selection and development with an indication of whether (YES) or not (NO) methyl jasmonate treatment had an effect on each stage. See text for further details

the establishment phase by helping to overcome tree resistance (Paine et al. 1997). Even though we did not evaluate the effects of our treatment on fungal colonization, other studies have shown that MJ treatment increases resistance to *C. polonica* and other pathogens in Norway spruce (Kozlowski et al. 1999; Franceschi et al. 2002; Schmidt et al. 2005; Zeneli et al. 2006). Thus, MJ treatment might well have affected beetle colonization via its effect on the fungal associates.

Methyl jasmonate application also reduced both the quality and quantity of brood production by *I. typographus*. These results agree with studies showing that bark beetles colonizing trees whose defenses had been induced usually experience increased levels of egg and larval mortality (Nebeker et al. 1993; Raffa and Smalley 1995). For example, *I. typographus* was less successful in attacking Norway spruce that had been elicited by a sublethal number of inoculations with the fungus *C. polonica* (Christiansen and Krokene 1999).

The dispersal phase includes offspring emergence from natal trees and response to host stimuli and/or aggregation pheromones. MJ application is likely to have negatively influenced the fitness and dispersal ability of emerging beetles by reducing their body weight and lipid content (Phase 4/Fig. 2). Although we did not determine lipid content directly, body weight and lipid content are directly correlated in *I. typographus* (Anderbrant et al. 1985). Lipids are used as an energy source during dispersal (Slansky and Haack 1986), and have been shown to affect bark beetle survival, host colonization, host selection and reproduction (Anderbrant et al. 1985; Anderbrant 1988; Wallin and Raffa 2004).

The anatomical and chemical changes induced by the application of MJ to *P. abies* in this study may be responsible for the increase in bark beetle resistance. MJ treatment has been suggested to increase resistance to herbivores and pathogens in previous studies in a similar manner (Kozlowski et al. 1999; Franceschi et al. 2002; Martin et al. 2002; Hudgins and Franceschi 2004; Zeneli et al. 2006). However, further investigation is required to confirm this conclusion, since MJ could also have acted by increasing other chemical or physical defenses that were not measured. In the current study, MJ induced the formation of numerous traumatic resin ducts in the sapwood, and so it can be assumed to have increased the volume of resin available for repelling biological invaders and sealing off wounds. An increase in traumatic resin ducts has been implicated in defense against *C. polonica*, a pathogenic fungal associate of *I. typographus* (Krokene et al. 2003; Zeneli et al. 2006).

MJ application also induced a 2.5–3-fold increase in the terpene content of Norway spruce. Conifer terpenes, which include monoterpenes, sesquiterpenes, and diterpene resin acids (Phillips and Croteau 1999), are thought to be an important constitutive defense against insect and pathogen attack (Berryman 1972; Raffa and Berryman 1983; Christiansen 1985). The terpene response to MJ treatment was negatively correlated with the degree of bark beetle colonization, since colonization was less successful in 2003, when the terpene response was strong, as compared to 2004, when the terpene response was weaker. The increased terpene levels lasted longer in the wood than in the bark, probably because much of the wood terpene is stored for a much longer time in the traumatic resin ducts and/or because the ducts continued to synthesize terpenes. Traumatic resin ducts may remain active for several years (Nagy et al. 2000). The terpene response in this study is consistent with a previous report on MJ treatment of mature *P. abies* (Zeneli et al. 2006).

The physical distribution and chemical content of traumatic resin ducts may provide a formidable obstacle to attack and growth of invasive organisms. However, traumatic resin duct development usually requires 2–4 weeks, which is not fast enough to repel a rapid attack of bark beetles (Franceschi et al. 2000; Nagy et al. 2000). Other more rapidly inducible defenses may function in this capacity. In addition to traumatic resin ducts, MJ application to conifers has also been shown to cause the activation and proliferation of polyphenolic parenchyma (PP) cells (Hudgins et al. 2003, 2004; Hudgins and Franceschi 2004) and to induce several enzymes of phenolic biosynthesis (Richard et al. 2000). The PP cells are primary sites of phenolic biosynthesis and other defense responses in the secondary phloem (Franceschi et al. 1998, 2000). However, in the current study MJ treatment did not cause any change in the content of soluble phenolic compounds. Similarly, Brignolas et al. (1998) found no changes in stilbene aglycones, a major group of soluble phenolic compounds, in fungus-inoculated phloem of *P. abies*. In any case, the defensive role of the stilbenes, flavonoids and other simple phenolics studied to date is ambiguous (Woodward and Pearce 1988; Evensen et al. 2000; McNee et al. 2003). The striking anatomical changes that take place in the PP cells after MJ application or fungal infection (Franceschi et al. 1998, 2000) may be associated with increases in more complex phenolics, such as high molecular weight condensed tannins (Maie et al. 2003) or cell wall-bound substances (Strack et al. 1988), but this remains to be tested (Bonello and Blodgett 2003; Blodgett et al. 2005).

Despite the clear-cut results of this study, we are very cautious about inferring that MJ can be a useful tool for protecting trees from bark beetle attacks. We do not yet know the impact of MJ on long-term tree health nor the costs of the anatomical and chemical changes induced by MJ application with respect to growth and survival. Nevertheless, further research on MJ-treated *P. abies* and *I. typographus* might help answer these questions as well as identify the precise defensive changes responsible for reduced *I. typographus* colonization, attraction and reproduction, and provide other important new insights about tree defences against bark beetles. If MJ treatment can be eventually shown to prevent tree mortality caused by bark beetles, this could be of great benefit to the integrated management of these important forest pests.

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