

This is an author produced version of a paper published in *Phytochemistry*. This paper has been peer-reviewed and is proof-corrected, but does not include the journal pagination.

Citation for the published paper:

Zhao, T., Krokene, P., Björklund, N., Långström, B., Solheim, H., Christiansen, E., Borg-Karlson, A-K. (2010) The influence of *Ceratocystis polonica* inoculation and methyl jasmonate application on terpene chemistry of Norway spruce, *Picea abies*.

Phytochemistry.

Volume: 71 Number: 11-12, pp 1332-1341.

<http://dx.doi.org/10.1016/j.phytochem.2010.05.017>

Access to the published version may require journal subscription.

Published with permission from: Elsevier



Epsilon Open Archive <http://epsilon.slu.se>

The influence of *Ceratocystis polonica* inoculation and methyl jasmonate application on terpene chemistry of Norway spruce, *Picea abies*

Tao Zhao ^a, Paal Krokene ^b, Niklas Björklund ^c, Bo Långström ^c,
Halvor Solheim ^b, Erik Christiansen ^b, Anna-Karin Borg-Karlson ^{a,*}

^a Ecological Chemistry Group, Department of Chemistry, Royal Institute of Technology, SE-100 44 Stockholm, Sweden

^b Norwegian Forest and Landscape Institute, N-1431 Ås, Norway

^c Department of Ecology, Swedish University of Agricultural Sciences, SE-750 07 Uppsala, Sweden

* Corresponding author. Tel.: + 46 8 7908449; fax: + 46 8 7912333.

E-mail address: akbk@kth.se

ABSTRACT

Constitutive and inducible terpene production is involved in conifer resistance against bark beetles and their associated fungi. In this study 72 Norway spruce (*Picea abies*) were randomly assigned to methyl jasmonate (MJ) application, inoculation with the bluestain fungus *Ceratocystis polonica*, or no-treatment control. We investigated terpene levels in the stem bark of the trees before treatment, 30 days and one year after treatment using GC-MS and two-dimensional GC (2D-GC) with a chiral column, and monitored landing and attack rates of the spruce bark beetle, *Ips typographus*, on the trees by sticky traps and visual inspection. Thirty days after fungal inoculation the absolute amount and relative proportion of (+)-3-carene, sabinene, and terpinolene increased and (+)- α -pinene decreased. Spraying the stems with MJ tended to generally increase the concentration of most major terpenes with minor alteration to their relative proportions, but significant increases were only observed for (-)- β -pinene and (-)-limonene. Fungal inoculation significantly increased the enantiomeric ratio of (-)- α -pinene and (-)-limonene one month after treatment, whereas MJ only increased that of (-)-limonene. One year after treatment, both MJ and fungal inoculation increased the concentration of most terpenes relative to undisturbed control trees, with significant changes in (-)- β -pinene, (-)- β -phellandrene and some other compounds. Terpene levels did not change in untreated stem sections after treatment, and chemical induction by MJ and *C. polonica* thus seemed to be restricted to the treated stem section. The enantiomeric ratio of (-)- α -pinene was significantly higher and the relative proportions of (-)-limonene were significantly lower in trees that were attractive to bark beetles compared to unattractive trees. One month after fungal inoculation, the total amount of diterpenes was significantly higher in putative resistant trees with shorter lesion lengths in response to fungal inoculation than in putative susceptible trees with longer lesions. Thus, terpene composition in the stem bark may be related to resistance of Norway spruce against *I. typographus* and *C. polonica*.

Keywords: *Picea abies*
Ceratocystis polonica
Methyl jasmonate
Terpene
Defense
Ips typographus

1. Introduction

Bark beetles and pathogenic fungi are major threats to conifer forests worldwide. The spruce bark beetle, *Ips typographus*, the economically most important insect pest of mature Norway spruce, *Picea abies* (Christiansen and Bakke, 1988), is associated with phytopathogenic blue-stain fungi such as *Ceratocystis polonica* (Solheim, 1986). This beetle typically reproduces in fallen trees and timber when the beetle population is below the epidemic threshold, but can also colonize living trees during epidemics (Christiansen and Bakke, 1988). Healthy conifers have effective constitutive and inducible defense mechanisms against invaders (Phillips and Croteau, 1999; Franceschi et al., 2005; Keeling and Bohlmann, 2006a; Gershenzon and Dudareva, 2007), but biotic and abiotic stressors such as windstorms and drought may predispose trees to *I. typographus* attack (Faccoli, 2009). In order to maintain healthy forests and develop practical methods to enhance tree resistance a deeper understanding of conifer defense mechanisms is necessary.

The potential to improve plant resistance through preparative pathogen infections is well documented in conifers (e.g. Christiansen et al., 1999; Eyles et al., 2010). In Norway spruce pre-inoculation of *C. polonica* enhances tree resistance to subsequent *C. polonica* infection (Christiansen et al., 1999; Krokene et al., 1999, 2001, 2003). More recently, methyl jasmonate (MJ), a vital cellular regulator that mediates diverse developmental processes in plants, has been demonstrated to alter defense responses against biotic and abiotic stresses in various plant species (Thaler, 1999). In Norway spruce MJ application reduces the colonization of *C. polonica* (Franceschi et al., 2002; Zeneli et al., 2006; Krokene et al., 2008) and the root pathogen *Pythium ultimum* (Kozłowski et al., 1999). MJ application also reduces tunneling and oviposition by *I. typographus* (Erbilgin et al., 2006).

MJ induces many of the same complex responses as pathogen infection in Norway spruce and other conifers, including traumatic resin duct formation, swelling of polyphenolic parenchyma cells (PP cells), enhanced resin flow (Franceschi et al., 2002; Hudgins et al., 2004), increased stem terpene concentrations (Martin et al., 2002; Erbilgin et al., 2006; Zeneli et al., 2006) and terpene emission from the foliage (Martin et al., 2003). Based on such observations MJ has been assumed to induce the same chemical response in conifers as fungal infection. However, there have been no detailed comparisons of the effect of fungal inoculation and exogenous MJ application on terpene chemistry in conifers.

In Sweden the storm Gudrun felled 75 million m³ of mostly Norway spruce trees on 8-9 January 2005 (Anonymous, 2006). The storm disturbance provided ample breeding material for *I. typographus*, and probably also reduced the resistance of many living trees. In the years following the storm, ca. 3 million m³ of standing spruce forests have been killed by the spruce bark beetle (Långström et al., 2009). The beetle outbreak represented a great

opportunity to learn more about the defense mechanisms in Norway spruce and to test methods to experimentally improve tree resistance. The aims of the present study were to investigate the effects of MJ application and *C. polonica* inoculation on the terpene chemistry of Norway spruce and how this influences host colonization by *I. typographus*.

2. Results

2.1. Terpene composition before treatment

Monoterpenes accounted for ca. 70% of total terpenes, with β -pinene and α -pinene as the dominant compounds, followed by β -phellandrene, limonene, myrcene and 3-carene in order of decreasing abundance (Table 1). The concentration of sesquiterpenes was low (< 8% of total terpenes), with (-)-germacrene D, β -caryophyllene, longifolene, germacrene D-4-ol, δ -cadinene, and α -longipinene as the major components. More than 10 diterpenes, including pimaric acid, methyl dehydroabietate, manoyl oxide, and dehydroabietic acid, were detected in the samples, but only thunbergol, abienol and neoabienol were present in relatively high concentrations. Both absolute amounts and relative proportions of terpenes varied among individual trees before treatment, but no significant differences were observed among trees assigned to different treatments (except for one minor compound, bornyl acetate that was ~2-fold more abundant in trees assigned to MJ treatment because three trees in this group had relatively high amounts of bornyl acetate) (Table 1).

The enantiomeric composition of α -pinene and limonene was highly variable, with the (-)-enantiomer making up 35.2 - 83.3% of the total in individual trees for α -pinene, and 28.2 - 93.2% for limonene. No significant differences were observed in mean enantiomeric composition of trees assigned to different treatments before treatment (Fig. 1). For β -pinene and β -phellandrene the (-)-enantiomer dominated, making up > 96% of the total, and the enantiomeric composition varied little between trees.

2.2. Terpene composition in the untreated stem section

To determine if *C. polonica* (Cp) or MJ treatment induced systemic terpene responses in the trees, samples were taken 30 cm below the treated stem section on all trees one month and one year after treatment. The concentrations of some compounds, such as (+)-3-carene, sabinene and thunbergol, were somewhat higher in trees treated with MJ or Cp than in control trees 30 days after treatment, but no significant differences were found outside the treated stem area for absolute amounts (Table 1), relative proportions or enantiomeric composition (Fig. 1) for any terpene, treatment or sampling date.

2.3. Changes in terpene composition in the treated stem section

2.3.1. One month after treatment

Both fungal inoculation and MJ application changed the terpene composition on day 30 compared with that on day 0. There were no significant changes in control trees over the same period. Trees from different treatments separated quite well in the PCA plot based on absolute amounts of terpenes on day 30, but trees treated with MJ or CT did not separate well in the plot based on relative amounts (Fig. 2).

Fungal inoculation significantly changed the amount of four terpenes (Table 1, Fig. 3). (+)-3-Carene showed the most remarkable change 30 days after treatment, with 1.8 - 23.2 fold increase in absolute amounts in the analyzed trees ($t = 4.83$, $p < 0.01$). The relative amount of (+)-3-carene also increased significantly compared to day 0 ($t = 5.86$, $p < 0.01$), and by day 30 it was one of the most abundant terpenes in many inoculated trees (data for relative amounts not shown). Inoculation also increased absolute (Table 1, Fig. 3) and relative amounts (not shown) of sabinene (ab: $t = 4.77$, $p < 0.01$; re: $t = 3.22$, $p = 0.01$) and terpinolene (ab: $t = 5.21$, $p < 0.01$; re: $t = 3.48$, $p < 0.01$), whereas (+)- α -pinene became significantly less abundant 30 days after inoculation (ab: $t = 2.83$, $p = 0.025$; re: $t = 2.58$, $p = 0.039$) (Fig. 3). No significant changes were observed on absolute or relative amounts of (-)- α -pinene, (-)- β -phellandrene, (+)-limonene, (-)-limonene (Fig. 3) or other terpenes (Table 1) 30 days after fungal inoculation ($p = 0.091 - 0.848$).

Compared with fungal inoculation, the terpene response to MJ treatment was more variable between trees. Among the 16 analyzed trees, total terpene levels increased 8.5 fold in one tree, 1.3 - 2.9 fold in eight trees, whereas seven trees showed very little change (data not shown). The absolute amount of (+)- α -pinene, (-)- β -pinene, (+)-limonene, (-)-limonene, (-)- β -phellandrene and most other terpenes tended to increase in the treated stem section after MJ application, but significant increases were only observed for (-)-limonene ($t = 2.53$, $p = 0.039$) and (-)- β -pinene ($t = 2.38$, $p = 0.048$) due to the high individual variation (Table 1, Fig. 3). The relative amounts of (+)-3-carene and (-)-limonene increased in some trees, but there was no significant change in relative amount of any terpene after MJ application (data not shown).

Fungal inoculation significantly increased the enantiomeric composition of both α -pinene ($t = 9.29$, $p < 0.01$) and limonene ($t = 3.81$, $p < 0.01$), while MJ treatment only significantly increased that of limonene ($t = 4.01$, $p < 0.01$). There were no significant enantiomeric changes for these terpenes in the control trees (Fig. 1). The enantiomeric composition of β -pinene and β -phellandrene did not change in any treatments (data not shown).

2.3.2. One year after treatment

The terpene profile of MJ and Cp treated trees varied notably from control trees one year after treatment, and thus separated those trees from controls in PCA plots (Fig. 4). Two Cp

treated trees (3c and 21c) and one MJ treated tree (14b) with greatly elevated terpene levels stood out from the others in the PCA plot (Fig. 4A). Most of the MJ treated trees and half the Cp treated trees had a terpene profile leaning towards an increase in (+)- and (-)- α -pinene, (-)- β -phellandrene, (-)- β -pinene, myrcene, and (+)-limonene. The remaining Cp treated trees tended to show an increase of 3-carene, terpinolene, sabinene and thunbergol. In general, the absolute amount of (-)- β -pinene ($F_{3,28} = 3.197, p = 0.039$) and (-)- β -phellandrene ($F_{3,28} = 3.094, p = 0.048$) differed significantly between treatments. Trees treated with fungus or MJ had significant higher amounts of (-)- β -pinene ($p < 0.01$ and $p = 0.048$, respectively) and (-)- β -phellandrene ($p = 0.012$ and $p = 0.028$) in the treated stem section than undisturbed control (TC) trees with no previous sampling history. In addition, Cp treated trees had significant higher levels of (-)-germacrene D ($p < 0.01$) and MJ treated trees had higher levels of δ -cadinene ($p < 0.01$) than the controls. No significant differences were observed between control trees with and without sampling history for any compound.

The relative amounts of some compounds also differed between treatments one year after treatment. Proportionally, (-)- α -pinene ($p = 0.018$ and $p < 0.01$ for Cp and MJ, respectively) and camphene ($p = 0.016$ and $p < 0.01$) were lower in Cp and MJ treated trees than in TC trees. (+)-3-Carene was more abundant in fungal inoculated trees than in MJ treated trees ($p = 0.018$) and control trees ($p < 0.01$). These differences separated most of the treated trees from the controls in the PCA plot based on relative amounts of all quantified terpenes (Fig. 4B). No differences in enantiomeric composition were observed between treatments one year after treatment.

2.4. Relationship between terpene composition, bark beetle attack and fungal performance

In 2007, beetle flight was monitored using pheromone traps in the vicinity of the study area. Since beetle flight had culminated before the trees were baited with pheromones, most of the trees had rather light attacks in 2007 with no significant differences between treatments (no. of landing beetles: $F_{2,69} = 0.23, p = 0.80$; no. of beetle entrance holes: $F_{2,69} = 1.78, p = 0.18$). In April 2008, two control trees and one tree each treated with Cp or MJ were recorded as dead from beetle attack in 2007. In 2008, beetle flight peaked in early May, and hence some beetles had flown before the trees were furnished with pheromones and sticky traps. Again, no significant differences in beetle attack were observed between treatments in 2008.

To investigate the relationship between terpenes and beetle attack more closely we selected six attractive and seven unattractive trees based on the number of beetles that had been landing on the sticky traps or entered the bark on the lower 4 m of the stem in 2007 (attractive trees had > 20 beetles landing on them and / or > 10 entrance holes up until 17 August; unattractive trees had < 5 beetles landing or entering). There were no significant differences between the two groups in absolute amounts of any terpene at any sampling date, but the relative amount of (-)-limonene was significantly higher in unattractive trees than in

attractive ones on both day 0 ($p = 0.047$) and day 30 ($p = 0.033$). Similarly, the enantiomeric composition of imonene was somewhat higher ($p = 0.12$ on day 0 and $p = 0.08$ on day 30), and that of α -pinene was significantly lower ($p = 0.046$ on day 0 and $p = 0.039$ on day 30) in unattractive trees than in attractive ones (Fig. 5).

Lesion lengths in the 24 Cp treated trees varied between 22 and 73 mm, indicating that the trees differed in their susceptibility to *C. polonica*. To investigate possible effects of terpenes on this fungus we compared terpene levels in putative susceptible trees with long lesions (> 50 mm, $n = 3$) and putative resistant trees with short lesions (< 30 mm, $n = 7$). No significant differences were found between the two groups before treatment, but one month after treatment the absolute amount of thunbergol and the total amount of all quantified diterpenes was significantly higher in putative resistant trees with shorter lesions (Table 2).

3. Discussion

Terpenes include many compounds that are toxic to insects and microorganisms, including monoterpenes such as limonene and 3-carene, and semi-crystalline diterpenes that polymerize to form a hardened barrier that seals wounds or traps insect invaders (Phillips and Croteau, 1999; Keeling and Bohlmann, 2006b; Seybold et al., 2006; Gershenzon and Dudareva, 2007). Different terpenes probably work synergistically to discourage attacking insects and kill or contain pathogens (Phillips and Croteau, 1999; Gershenzon and Dudareva, 2007). Our results show that both *C. polonica* inoculation and MJ application change the terpene content of Norway spruce bark in ways that may directly or indirectly influence the suitability of the trees to *I. typographus* and *C. polonica*.

In this experiment, the terpene response to MJ treatment and *C. polonica* inoculation differed extensively one month after treatment. Fungal inoculation seemed to induce mainly qualitative responses, i.e. it only affected levels of (+)-3-carene, sabinene, terpinolene and (+)- α -pinene, but did not notably change other terpenes. MJ on the other hand tended to induce quantitative responses in Norway spruce terpenes with minor effects on relative proportions (although significant quantitative responses were only observed for (-)- β -pinene and (-)-limonene due to large individual variation). We can distinguish between a MJ-specific induction profile characterized by an increase in (-)-limonene and (-)- β -pinene, and a fungus-specific induction profile with an increase in (+)-3-carene, terpinolene and sabinene and a decrease in (+)- α -pinene. This suggests that *C. polonica* and MJ induce different changes at a detailed chemical level, probably because they activate different biochemical pathways. Cell culture studies with Norway spruce also indicate that methyl jasmonate and *C. polonica* have differential effects on enzymes involved in defense related terpene biosynthesis. Methyl jasmonate upregulates one isoform of 1-deoxy-D-xylulose 5-phosphate synthase (DXS), an enzyme catalyzing the first step in the methylerythritol

phosphate (MEP) pathway, whereas treatment with *C. polonica* or methyl salicylate upregulates another isoform (Phillips et al., 2007).

(+)-3-Carene, sabinene and terpinolene are products of same multi-product enzyme in Norway spruce (Fäldt et al., 2003) and their quantities were found to be closely correlated in our study. Interestingly, two compounds that tended to decrease one month after fungal inoculation, (+)- α -pinene and (+)-limonene, were also positively correlated (Fig. 6), suggesting that they also may be produced by the same terpene synthase or by different, co-expressed enzymes. These results indicate that fungal infection upregulates (+)-3-carene synthase in Norway spruce, inhibits other terpene synthases, whereas some does not seem to be affected at all.

Terpene accumulation following MJ treatment has been observed repeatedly in Norway spruce (Erbilgin et al., 2006; Zeneli et al., 2006; Zulak et al., 2009). Zulak et al. (2009) measured the induction and activity of terpene synthases in a single MJ treated Norway spruce clone. Their results basically agree with our findings, except that they observed a strong induction of (+)-3-carene synthase up to 32 days after treatment. Erbilgin et al. (2006) showed that monoterpene, diterpene and total terpene levels were significantly higher in MJ treated bark than in control tissue. They suggested that this chemical induction could be directly responsible for the observed decrease in *I. typographus* colonization and reproduction in MJ treated trees. More specifically, in a multi-clone experiment, Zeneli et al. (2006) demonstrated that the concentration of MJ needed to trigger terpene accumulation, the speed of the response and the extent of terpene accumulation vary extensively among Norway spruce clones. We also found considerable variation in response to MJ application among individual Norway spruce trees. Half of our trees did not respond at all, while the other half responded strongly. The variable induction may reflect genuine differences in MJ sensing or signaling systems among the trees or may simply result from differences in the ability of MJ to penetrate the outer bark (Zeneli et al., 2006).

Long lasting terpene induction after MJ treatment has been observed earlier (Erbilgin et al., 2006), but no such effect has previously been reported one year after inoculation with *C. polonica*. The terpene response of Norway spruce to fungal inoculation tended to be stronger and more diverse one year after treatment than after one month. This might be due to the expansion over time of the necrotic lesion induced by fungal infection in the bark and that our samples thus were collected closer to the lesion after one year. The so called reaction zone induced by fungal infection is known to contain much higher levels of terpenoids than normal phloem (Viiri et al., 2001; Fäldt et al., 2006). We might speculate that Norway spruce trees employ different chemical strategies at different stages of fungal invasion. The increased biosynthesis of (+)-3-carene, terpinolene and sabinene at a cost of decreased (+)- α -pinene and (+)-limonene production observed one month after inoculation may be the

first step in the chemical response to infection, serving as a prevention strategy in phloem outside the area colonized by the fungus. A more quantitative chemical response, which obviously is more energy demanding, may be the next step as the fungus gradually is approaching the sampling position.

Anatomical studies of Norway spruce have shown that wounding, fungal infection and MJ treatment induce traumatic resin duct formation in more remote stem tissues (Franceschi et al., 2000; Krokling et al., 2004; Krokene et al., 2008), and systemic chemical changes in e.g. phenolics have been observed in other conifers (Bonello and Blodgett, 2003), demonstrating that conifers possess systemically inducible signalling pathways. However, in the present experiment chemical changes were restricted to the pretreated areas of the stem. No chemical changes were induced in the untreated lower stem section, which is in agreement with the observation that *C. polonica* pretreatment did not enhance tree resistance to infection outside the pretreated stem section (Krokene et al., 1999).

Despite distinct chemical changes in the treated stem sections indicating up- or down-regulated chemical defense reactions in the trees, we found no significant differences in landing or entry rates by the spruce bark beetle in either inoculated or MJ treated trees. This is in contrast with the findings of Ergilbin et al. (2006), who demonstrated increased terpene levels and reduced spruce bark beetle attacks after MJ treatment of Norway spruce. Similarly, sublethal fungal inoculation has been shown to reduce spruce bark beetle performance in Norway spruce (Christiansen and Krokene, 1999). A possible explanation for our results may be that we missed the major part of the flight season in 2007, when the main beetle flight took place in late April, several weeks earlier than normal. In mid-June, when our experiment started, the main flight was over and there were already callow adults under the bark of trap logs at Tönnersjöheden Experimental Forest (B. Långström, unpubl. data). Hence, we mainly attracted sister-brood-flyers in low numbers to our experimental trees, resulting in insufficient attack rates to overcome tree resistance. Only four of the 72 experimental trees were successfully colonized and killed by the beetles, whereas 11 out of 20 untreated, pheromone-baited control trees were killed in a follow - up study in the same stand in 2008 (P. Krokene, unpubl. data).

Induction of 3-carene is repeatedly observed after fungal infection or MJ treatment in conifers. For example, 3-carene is the major monoterpene induced in Scots pine roots upon infection with the ectomycorrhizal fungus, *Boletus variegatus* (Krupa and Fries, 1971). Infection of *Grosmannia clavigera* in lodgepole pine (Croteau et al., 1987) and *Leptographium wingfieldii* in Scots pine (Fäldt et al., 2006) phloem also induce high 3-carene production. Significantly higher (+)-3-carene accumulation has also been observed in Norway spruce saplings after MJ treatment (Zulak et al., 2009). Previous investigations have already associated 3-carene levels with conifer resistance or susceptibility to fungi or insects

(Reed et al., 1986; Rocchini et al., 2000; Pasquier-Barre et al., 2001), indicating that 3-carene may be a useful chemical marker of conifer resistance.

The enantiomers of plant terpenes may be important for insect-host interactions (Stranden et al., 2002, 2003; Mustaparta and Stranden, 2005). Olfactory receptor neurons in the large pine weevil *Hylobius abietis* respond more strongly to (+)- α -pinene and (-)-limonene than to their opposite enantiomer (Wibe et al., 1998). The common pine shoot beetle *Tomicus piniperda* preferred to enter shoots of trees that emitted higher proportions of (-)- α -pinene (Almquist et al., 2006). Similarly, *I. typographus* had significantly stronger electroantennogram responses to (-)- α -pinene than to the (+)-enantiomer (Dickens, 1978), and (-)- α -pinene also increased the attraction of *I. typographus* to its aggregation pheromone in a field experiment (Erbilgin et al., 2007), indicating that (-)- α -pinene is attractive to *I. typographus*. More interesting, since *I. typographus* utilizes (-)- α -pinene as a precursor to biosynthesize its pheromone component cis-verbenol, the enantiomeric composition of α -pinene in the host may directly influence the production of aggregation pheromones (Birgersson et al., 1984). In this study we found that attractive Norway spruce trees had a significantly higher enantiomeric proportion of (-)- α -pinene and a somewhat lower enantiomeric proportion of (-)-limonene than unattractive trees. This suggests that the enantiomeric composition of α -pinene and limonene may directly influence the suitability of Norway spruce to *I. typographus*. *C. polonica* inoculation increased the ratio of (-)-enantiomers of α -pinene and limonene, and MJ application increased that of limonene one month after treatment. This may alter the resistance of Norway spruce, but it is difficult to speculate further on the ecological significance of these changes since beetle attack rates were relatively low in our experiment.

4. Concluding remarks

This study demonstrated that *C. polonica* inoculation and MJ treatment induced chemical changes in Norway spruce bark. The induction profile of the two treatments differed markedly one month after treatment, suggesting that they activate different signal transduction pathways that affect terpene biosynthesis in different ways. Thus, treatment with MJ may not always be a perfect mimic for e.g. fungal infection when studying conifer defence responses. However, the full biological significance of these chemical changes is still not fully understood and further research needs to be done.

5. Experimental

5.1. Field procedures

The experiment was carried out in a pure stand of 47 - year - old Norway spruce that was planted in 1964 using 4 - year - old seedlings at Tönnersjöheden Experimental Forest (56°

41° N, 13°4' E), Halland, Sweden. On 10 - 11 May 2007, 24 triplets of neighboring trees (mean diameter at 1.3 m height: 218 mm; range 174 - 253 mm) were randomly assigned to either *C. polonica* (Cp), methyl jasmonate (MJ), or control (CT) treatment, with eight triplets (24 trees) per treatment. The Cp trees had the lower parts of their stems (0.8 to 3.8 m above ground) inoculated with *C. polonica* at a density of 20 per m² bark surface, using a 5 mm cork borer. Inoculum consisted of mycelium that had been growing on malt agar (2% malt, 1.5% agar) for one week. The strain used was NFLI 1993 - 208 / 115, which was isolated from a Norway spruce log inoculated with the bark beetle *Polygraphus poligraphus* L. (Krokene and Solheim, 1996). The strain has been used in several previous inoculation studies (e.g. Christiansen et al., 1999; Nagy et al., 2004; Krokene et al., 2001, 2003; Zeneli et al., 2006). On trees assigned to MJ treatment the lower stem (0.8 to 3.8 m) was sprayed with 100 mM MJ in water with 0.1% Tween 20. The bark was kept wet for a minimum of five minutes by repeated application of MJ, and CT trees received no treatment.

On 11 June, a 10 cm long pheromone dispenser tape (Hercon ® type) was attached at 1.0 m height on the SW side of all trees. An additional 50 cm dispenser tape was placed at the same height on a pole in the center of each triplet. To monitor *I. typographus* landing rates on the trees one sticky trap (Pherobank ®, 10 × 15 cm) was placed at 1.3 m height on the SW side of each tree on 11 June and inspected repeatedly until 17 August 2007. We also determined the number of beetle entrance holes on the lower 4 m of the stem by visual inspection.

In spring 2008, the surviving study trees were again furnished with pheromones and sticky traps. On 6 May, new pheromone dispensers and sticky traps were put on the stick in the center of each triplet of trees. On 21 May, all trees were visually inspected between 1 - 2 m stem height, and the number of beetle attacks was recorded. Beetles were counted and removed from all sticky traps on 27 May, 11 and 25 June, and 28 August.

Lesion lengths on *C. polonica* treated trees were measured 19 - 20 October 2009. The outer bark around each inoculation point was removed and maximum length of visible necrotic lesions was measured at the outer surface of the phloem.

5.2. Bark sampling and sample extraction

In 2007, bark samples for chemical analyses were taken from all experimental trees on the day of treatment (10 - 11 May, day 0) and one month later (10 - 11 June, day 30) using a 5 mm cork borer. Five additional unattacked trees of similar size within the experimental stand were selected and sampled on day 30. Samples consisted of single bark plugs taken from the four cardinal points of each tree and pooled into a single sample per day. On day 0, immediately before stem treatment with MJ or Cp, samples were taken 1.3 m above ground. On day 30, one sample was taken 1.3 m above ground to observe induced chemical changes

in the treated stem section, and another at 0.5 m to observe possible systemic changes induced by the treatments. On trees that had been inoculated with *C. polonica*, samples in the treated section were taken 5 cm above an inoculation point. To reduce the influence of previous sampling at the same height, new samples were always taken as far away as possible from previous sampling sites.

Eight trees with notable chemical induction from each of the Cp- and MJ- treated groups and eight randomly chosen control trees were re-sampled approximately one year later (day 362), to determine the durability of the chemical induction. In addition, eight unattacked trees of similar size within the experimental stand were also selected and sampled on that day, serving as undisturbed true control trees (TC).

The outer cork bark was removed from the bark plugs and the plugs were submerged in 1.0 ml of hexane containing 0.30 mg pentadecane (Lancaster synthesis, England) as an internal standard and 0.12 mg 3-tert-butyl-4-hydroxy-anisol (Fluka, Switzerland) as antioxidant. The samples were extracted in hexane at room temperature for 48 h before the extract was transferred to new vials and kept at $-25\text{ }^{\circ}\text{C}$ until GC-MS and 2D-GC analyses. The bark plugs were dried at $80\text{ }^{\circ}\text{C}$ for 6 h, and then weighted on a Sartorius electronic balance for absolute amount calculation.

5.3. Terpene separation, identification and quantification

The hexane extracts were analyzed by a Varian 3400 GC connected to a Finnigan SSQ 7000 MS to separate, identify and quantify the terpene constituents. A SPB-1 fused silica capillary column (Supelco, 30 m, 0.25 mm i.d., and 0.25 μm film thickness) was used, and the temperature program was set as $40\text{ }^{\circ}\text{C}$ for 1 min, increasing to $230\text{ }^{\circ}\text{C}$ at a rate of $4\text{ }^{\circ}\text{C min}^{-1}$, and then remaining constant at $230\text{ }^{\circ}\text{C}$ for 19 min. A split / splitless injector was used with a 30 s splitless injection at $225\text{ }^{\circ}\text{C}$. The temperature of the transfer line was set at $235\text{ }^{\circ}\text{C}$. Helium was used as the carrier gas at a constant flow of 1 ml min^{-1} , the temperature of the ion source was $150\text{ }^{\circ}\text{C}$, the mass detector was operated with a mass range of 30 - 400, and the electron impact ionization was 70 eV. One μl hexane extract of each sample was injected into the GC-MS manually by using a 5 μl syringe. The terpene hydrocarbons were identified by comparing retention times and mass spectra with available authenticated standards, or by comparing retention indexes (RIs) and mass spectra with Massfinder 3 (Hochmuth Scientific Consulting, Germany) and the reference libraries of NIST (National Institute of Standards and Technology). The absolute amounts of terpenes were calculated relative to the internal standards and expressed as mg (or μg) g^{-1} dry wt. and expressed as pentadecan equivalents. The relative amounts of terpenes were calculated as the ratio of the area of each peak to the sum of all the areas of terpene hydrocarbons in a defined GC fraction, and expressed as percentages.

The enantiomeric composition of the four major monoterpenes (α -pinene, β -pinene, β -phellandrene and limonene) was analyzed by 2D-GC. 3-Carene was considered to be present as pure (+)-enantiomer and germacrene D as pure (-)-enantiomer according to previous studies (Persson et al., 1996; Strandén et al., 2003). Ten μ l hexane extract was added to a filter paper (size: 2 mm \times 20 mm) within a 3.5 ml vial, the solvent was evaporated for 30 s, and the vial was sealed with aluminum foil and equilibrated for 1 min. A SPME fiber (65 μ m PDMS - DVB coating, Supelco, USA) was used to trap the volatiles in the headspace for 10 min at room temperature and then injected directly into the 2D-GC. The 2D-GC system consisted of two Varian 3400 GCs with flame ionization detectors (FID). The first GC was equipped with a DB-Wax column (J & W Scientific TM, 30 m, 0.25 mm i.d. and 0.25 mm film thicknesses). Volatiles were separated by the following temperature program: 40 °C for 1 min, increasing at a rate of 7 °C min⁻¹ to 220 °C, and then remaining at 220 °C for 5 min. Injections were performed with an injector temperature of 220 °C in 30 s of splitless mode. Enantiomeric proportions of the four selected monoterpenes were analyzed by sending the components into the second GC, which was equipped with a β -cyclodextrin column (J & W Scientific TM, 30 m \times 0.25 mm \times 0.25 μ m). The enantiomers were separated with a temperature program starting from 60 °C for 0.1 min, followed by 0.5 °C min⁻¹ up to 90 °C. Helium was used as the carrier gas at a pressure of 34 psi for the first GC and 22 psi for the second GC. The temperature of the FI-detectors was 225 °C. The enantiomeric composition of a specific enantiomer was defined as the percentage of (-)- enantiomer to the sum of the (+)- and (-)- enantiomers of the respective monoterpene. The absolute amount of the enantiomer was calculated by multiplying the proportion of the enantiomer by the absolute amount of the respective monoterpene obtained by GC-MS.

5.4. Data analyses

The absolute amounts and relative proportions (normalized to 100%) of all the quantified compounds were subjected to Principal Components Analysis (PCA) to evaluate the influence of treatments on terpene composition, using the multivariate data analysis software Canoco 4.5 (Biometris Plant Research International, The Netherlands). Changes in individual compounds between time points were compared by paired t-tests ($n = 16$ for nonchiral data, and $n = 8$ for chiral data) and the differences among treatments were tested by one way ANOVA. If treatments were significantly different ($p < 0.05$), means were separated using LSD at $p = 0.05$ (Statistica 6.0, Statsoft, Inc. USA). Correlation analysis was conducted by means of Pearson product-moment correlation coefficient. The relative proportions of terpenes were arcsin-transformed and the absolute amount data were square-root transformed to correct for unequal variance and departures from normality.

Acknowledgements

This study was supported by a special grant for bark beetle research from The Swedish University of Agricultural Sciences (SLU). In addition, The Swedish Research Council Formas and Norwegian Forest and Landscape Institute provided financial support. The authors thank Astrid Kännaste for technical assistance in 2D-GC analysis, and Karolin Axelsson, Morgan Erixon and Ulf Johansson for skilful assistance in the field.

References

- Almquist, A.C., Fäldt, J., Yart, A., Chevet, Y., Sauvard, D., Lieutier, F., Borg-Karlson, A.K., 2006. Host selection in *Tomicus piniperda* L.: composition of monoterpene hydrocarbons in relation to attack frequency in the shoot feeding phase. *Z. Naturforsch C.* 61, 439–444.
- Anonymous, 2006. Stormen 2005-en skoglig analys. Meddelande 1, 1–199 (In Swedish only).
- Birgersson, G., Schlyter, F., Löfqvist, J., Bergström, G., 1984. Quantitative variation of pheromone components in the spruce bark beetle *Ips typographus* from different attack phases. *J. Chem. Ecol.* 10, 1029–1055.
- Bonello, P., Blodgett, J.T., 2003. *Pinus nigra*–*Sphaeropsis sapinea* as a model pathosystem to investigate local and systemic effects of fungal infection of pines. *Physiol. Mol. Plant Pathol.* 63, 249–261.
- Christiansen, E., Bakke, A., 1988. The spruce bark beetle of Eurasia, In: Berryman, A.A. (Ed.), *Dynamics of Forest Insect Populations*. Plenum Publishing Corp., New York, pp. 479–503.
- Christiansen, E., Krokene, P., 1999. Can Norway spruce trees be “vaccinated” against attack by *Ips typographus*? *Agric. Forest Entomol.* 1, 185–187.
- Christiansen, E., Krokene, P., Berryman, A.A., Franceschi, V.R., Krekling, T., Lieutier, F., Lönneborg, A., Solheim, H., 1999. Mechanical injury and fungal infection induce acquired resistance in Norway spruce. *Tree Physiol.* 19, 399–403.
- Croteau, R., Gurkewitz, S., Johnson, M.A., Fisk, H.J., 1987. Biochemistry of oleoresinosis: Monoterpene and diterpene biosynthesis in lodgepole pine saplings infected with *Ceratocystis clavigera* or treated with carbohydrate elicitors. *Plant Physiol.* 85, 1123–1128.
- Dickens, J.C., 1978. Behavioural and electrophysiological responses of the bark beetle, *Ips typographus*, to potential pheromone components. *Physiol. Entomol.* 6, 251–261.
- Erbilgin, N., Krokene, P., Christiansen, E., Zeneli, G., Gershenzon, J., 2006. Exogenous application of methyl jasmonate elicits defenses in Norway spruce (*Picea abies*) and reduces host colonization by the bark beetle *Ips typographus*. *Oecologia* 148, 426–436.

- Erbilgin, N., Krokene, P., Kvamme, T., Christiansen, E., 2007. A host monoterpene influences *Ips typographus* (Coleoptera: Curculionidae, Scolytinae) responses to its aggregation pheromone. *Agric. Forest Entomol.* 9, 135–140.
- Eyles, A., Bonello, P., Ganley, R., Mohammed, C., 2010. Induced resistance to pests and pathogens in trees. *New Phytol.* 185, 893–908.
- Faccoli, M., 2009. Effect of weather on *Ips typographus* (Coleoptera Curculionidae) phenology, voltinism, and associated spruce mortality in the southeastern Alps. *Environ. Entomol.* 38, 307–316.
- Fäldt, J., Martin, D., Miller, B., Rawat, S., Bohlmann, J., 2003. Traumatic resin defense in Norway spruce (*Picea abies*): Methyl jasmonate-induced terpene synthase gene expression, and cDNA cloning and functional characterization of (+)-3-carene synthase. *Plant Mol. Biol.* 51, 119–133.
- Fäldt, J., Solheim, H., Långström, B., Borg-Karlson, A.K., 2006. Influence of fungal infection and wounding on contents and enantiomeric compositions of monoterpenes in phloem of *Pinus sylvestris*. *J. Chem. Ecol.* 32, 1779–1795.
- Franceschi, V.R., Krokene, P., Krekling, T., Christiansen, E., 2000. Phloem parenchyma cells are involved in local and distant defense responses to fungal inoculation or bark-beetle attack in Norway spruce (Pinaceae). *Am. J. Bot.* 87, 314–326.
- Franceschi, V.R., Krekling, T., Christiansen, E., 2002. Application of methyl jasmonate on *Picea abies* (Pinaceae) stems induces defense-related responses in phloem and xylem. *Am. J. Bot.* 89, 578–586.
- Franceschi, V.R., Krokene, P., Christiansen, E., Krekling T., 2005. Anatomical and chemical defenses of conifer bark against bark beetles and other pests. *New Phytol.* 167, 353–376.
- Gershenson, J., Dudareva, N., 2007. The function of terpene natural products in the natural world. *Nat. Chem. Biol.* 3, 408–414.
- Hudgins, J.W., Franceschi, V.R., 2004. Methyl jasmonate-induced ethylene production is responsible for conifer phloem defense responses and reprogramming of stem cambial zone for traumatic resin duct formation. *Plant Physiol.* 135, 2134–2149.
- Keeling, C.I., Bohlmann, J., 2006a. Genes, enzymes, and chemicals of terpenoid diversity in the constitutive and induced defence of conifers against insects and pathogens. *New Phytol.* 170, 657–675.
- Keeling, C.I., Bohlmann, J., 2006b. Diterpene resin acid in conifers. *Phytochemistry* 67, 2415–2423.
- Kozłowski, G., Buchala, A., Métraux, J.P., 1999. Methyl jasmonate protects Norway spruce (*Picea abies* (L.) Karst.) seedlings against *Pythium ultimum* Trow. *Physiol. Mol. Plant Pathol.* 55, 53–58.

- Krekling, T., Franceschi, V.R., Krokene, P., Solheim, H., 2004. Differential anatomical response of Norway spruce stem tissues to sterile and fungus infected inoculations. *Trees* 18, 1–9.
- Krokene, P., Solheim, H., 1996. Fungal associates of five bark beetle species colonizing Norway spruce. *Can. J. Forest Res.* 26, 2115–2122
- Krokene, P., Christiansen, E., Solheim, H., Franceschi, V.R., Berryman, A.A., 1999. Induced resistance to pathogenic fungi in Norway spruce. *Plant Physiol.* 121, 565–570.
- Krokene, P., Solheim, H., Christiansen, E., 2001. Induction of disease resistance in Norway spruce (*Picea abies*) by necrotizing fungi. *Plant Pathol.* 50, 230–233.
- Krokene, P., Solheim, H., Krekling, T., Christiansen, E., 2003. Inducible anatomical defense responses in Norway spruce stems and their possible role in induced resistance. *Tree Physiol.* 23, 191–197.
- Krokene, P., Nagy, N.E., Solheim, H., 2008. Methyl jasmonate and oxalic acid treatment of Norway spruce: anatomically based defense responses and increased resistance against fungal infection. *Tree Physiol.* 28, 29–35.
- Krupa, S., Fries, N., 1971. Studies on ectomycorrhizae of pine I. Production of volatile organic compounds. *Can. J. Bot.* 49, 1425–1431.
- Långström, B., Lindelöw, Å., Schroeder, M., Björklund, N., Öhrn, P., 2009. The spruce bark beetle outbreak in Sweden following the January-storms in 2005 and 2007, In: Kunca, A., Zubrik, M. (Eds.), *Insects and fungi in storm areas*. Proceedings of the IUFRO Working Party 7.03.10, Slovakia, pp. 13–19.
- Martin, D., Tholl, D., Gershenzon, J., Bohlmann, J., 2002. Methyl jasmonate induces traumatic resin ducts, terpenoid resin biosynthesis, and terpenoid accumulation in developing xylem of Norway spruce stems. *Plant Physiol.* 129, 1003–1018.
- Martin, D.M., Gershenzon, J., Bohlmann, J., 2003. Induction of volatile terpene biosynthesis and diurnal emission by methyl jasmonate in foliage of Norway spruce. *Plant Physiol.* 132, 1586–1599.
- Mustaparta, H., Strandén, M., 2005. Olfaction and learning in moth and weevils living on angiosperm and gymnosperm hosts. *Recent. Adv. Phytochem.* 39, 269–292.
- Nagy, N.E., Fossdal, C.G., Krokene, P., Krekling, T., Lönneborg, A., Solheim, H., 2004. Induced responses to pathogen infection in Norway spruce phloem: changes in polyphenolic parenchyma cells, chalcone synthase transcript levels and peroxidase activity. *Tree Physiol.* 24, 505–515.
- Passquier-Barre, F., Palasse, C., Goussard, F., Auger-Rozenberg, M.A., Geri, C., 2001. Relationship of Scots pine clone characteristics and water stress to hatching and larval performance of the sawfly *Diprion pini* (Hymenoptera: Diprionidae). *Environ. Entomol.* 30, 1–6.

- Persson, M., Sjödin, K., Borg-Karlson, A.K., Norin, T., Ekberg, I., 1996. Relative amounts and enantiomeric compositions of monoterpene hydrocarbons in xylem and needles of *Picea abies*. *Phytochemistry* 42, 1289–1297.
- Phillips, M.A., Croteau, R.B., 1999. Resin-based defense in conifers. *Trends Plant Sci.* 4, 184–190.
- Phillips, M.A., Walter, M.H., Ralph, S.G., Dabrowska, P., Luck, K., Urós, E.M., Boland, W., Strack, D., Rodríguez-Concepción, M., Bohlmann, J., Gershenzon, J., 2007. Functional identification and differential expression of 1-deoxy- D-xylulose 5-phosphate synthase in induced terpenoid resin formation of Norway spruce (*Picea abies*). *Plant Mol. Biol.* 65, 243–257.
- Reed, A.N., Hanover, J.W., Furniss, M.M., 1986. Douglas-fir and western larch: chemical and physical properties in relation to Douglas-fir bark beetle attack. *Tree Physiol.* 1, 277–287.
- Rocchini, L.A., Lindgren, B.S., Bennett, R.G., 2000. Effects of resin flow and monoterpene composition on susceptibility of lodgepole pine to attack by the Douglas-fir pitch moth, *Synanthedon novaroensis* (Lep., Sesiidae). *J. Appl. Entomol.* 124, 87–92.
- Seybold, S.J., Huber, D.P.W., Lee, J.C., Graves, A.D., Bohlmann, J., 2006. Pine monoterpenes and pine bark beetles: a marriage of convenience for defense and chemical communication. *Phytochem. Rev.* 5, 143–178.
- Solheim, H., 1986. Species of Ophiostomataceae isolated from *Picea abies* infested by the bark beetle *Ips typographus*. *Nord. J. Bot.* 6, 199–207.
- Stranden, M., Borg-Karlson, A.K., Mustaparta, H., 2002. Receptor neuron discrimination of the germacrene D enantiomers in the moth *Helicoverpa armigera*. *Chem. Senses* 27, 143–152.
- Stranden, M., Liblikas, I., König, W.A., Almaas, T.J., Borg-Karlson, A.-K., Mustaparta, H., 2003. Germacrene D receptor neurones in three species of heliothine moths: structure–activity relationships. *J. Comp. Physiol. A* 189, 563–577.
- Thaler, J.S., 1999. Jasmonic acid mediated interactions between plants, herbivores, parasitoids, and pathogens: A review of field experiments in tomato, In: Agrawal, A.A., Tuzun S., Bent E. (Eds.), *Induced plant defenses against pathogens and herbivores: biochemistry, ecology, and agriculture*. APS Press, St. Paul, MN, pp. 319–334.
- Viiri, H., Annala, E., Kitunen, V., Niemelä, P., 2001. Induced responses in stilbenes and terpenes in fertilized Norway spruce after inoculation with blue-stain fungus, *Ceratocystis polonica*. *Tree* 15, 112–122.
- Wibe, A., Borg-Karlson, A.K., Persson, M., Norin, T., Mustaparta, H., 1998. Enantiomeric composition of monoterpene hydrocarbons in some conifers and receptor neuron discrimination of α -pinene and limonene enantiomers in the pine weevil. *J. Chem. Ecol.* 24, 273–287.

Zeneli, G., Krokene, P., Christiansen, E., Krekling, T., Gershenzon, J., 2006. Methyl jasmonate treatment of mature Norway spruce (*Picea abies*) trees increases the accumulation of terpenoid resin components and protects against infection by *Ceratocystis polonica*, a bark beetle-associated fungus. *Tree Physiol.* 26, 977–988.

Zulak, K.G., Lippert, D.N., Kuzyk, M.A., Domanski, D., Chou, T., Borchers, C.H., Bohlmann, J., 2009. Targeted proteomics using selected reaction monitoring reveals the induction of specific terpene synthases in a multi-level study of methyl jasmonate-treated Norway spruce (*Picea abies*). *Plant J.* 60, 1015–1030.

Table 1. Absolute amounts of major terpene hydrocarbons ($\mu\text{g g}^{-1}$ dry wt equivalent to pentadecane) in Norway spruce phloem before (day 0) and after (day 30) stem treatment with methyl jasmonate (MJ) or inoculation with the bluestain fungus *Ceratocystis polonica* (CT) are untreated control trees). Samples were taken at 1.3 m stem height within the treated stem section (TS), but on day 30 an additional sample was taken 0.3 m below the treated stem section (UTS) at 0.5 m height. Data are expressed as means \pm 1 SD, $n = 16$ trees.

Compound	RI ²	<i>C. polonica</i>						MJ						CT					
		Day 0		Day 30		Day 30		Day 0		Day 30		Day 0		Day 30		Day 0		Day 30	
		TS	UTS	TS	UTS	TS	UTS	TS	UTS	TS	UTS	TS	UTS	TS	UTS	TS	UTS	TS	UTS
Tricyclene	918	2.0 \pm 0.3	2.1 \pm 0.3	2.2 \pm 0.2	2.1 \pm 0.4	2.3 \pm 0.3	2.1 \pm 0.2	2.5 \pm 0.5	2.3 \pm 0.3	2.1 \pm 0.2	2.7 \pm 0.2	2.0 \pm 0.4	2.0 \pm 0.4	2.0 \pm 0.4	2.7 \pm 0.2	2.0 \pm 0.4	2.7 \pm 0.2	2.0 \pm 0.4	2.0 \pm 0.4
α -Thujene	924	1.2 \pm 0.2	1.5 \pm 0.5	0.4 \pm 0.2	0.9 \pm 0.2	0.8 \pm 0.5	0.4 \pm 0.2	1.1 \pm 0.2	0.8 \pm 0.5	0.4 \pm 0.2	1.2 \pm 0.6	0.6 \pm 0.3	0.6 \pm 0.3	1.1 \pm 0.2	0.6 \pm 0.3	1.2 \pm 0.6	0.6 \pm 0.3	0.6 \pm 0.3	0.6 \pm 0.3
α -Pinene ¹	933	436.6 \pm 44.4	370.7 \pm 63.3	466.3 \pm 36.4	438.2 \pm 22.0	537.3 \pm 37.7	420.1 \pm 22.0	507.8 \pm 36.1	537.3 \pm 37.7	420.1 \pm 22.0	454.4 \pm 30.0	484.1 \pm 82.6	484.1 \pm 82.6	507.8 \pm 36.1	484.1 \pm 82.6	454.4 \pm 30.0	484.1 \pm 82.6	484.1 \pm 82.6	484.1 \pm 82.6
Camphene	943	8.7 \pm 0.9	7.0 \pm 1.7	8.1 \pm 0.7	9.8 \pm 2.4	9.2 \pm 1.9	7.7 \pm 0.6	11.2 \pm 2.8	9.2 \pm 1.9	7.7 \pm 0.6	9.2 \pm 2.0	7.4 \pm 1.6	7.4 \pm 1.6	11.2 \pm 2.8	7.4 \pm 1.6	9.2 \pm 2.0	7.4 \pm 1.6	7.4 \pm 1.6	7.4 \pm 1.6
Sabinene	967	6.6 \pm 0.6	15.6 \pm 1.0	5.3 \pm 0.2	6.9 \pm 0.7	6.3 \pm 0.5	6.4 \pm 0.3	5.0 \pm 0.8	6.3 \pm 0.5	6.4 \pm 0.3	6.3 \pm 1.2	4.3 \pm 1.6	4.3 \pm 1.6	5.0 \pm 0.8	4.3 \pm 1.6	6.3 \pm 1.2	4.3 \pm 1.6	4.3 \pm 1.6	4.3 \pm 1.6
β -Pinene ¹	971	480.8 \pm 53.3	516.3 \pm 48.8	422.3 \pm 43.1	494.2 \pm 25.9	707.1 \pm 96.3	422.3 \pm 51.8	424.9 \pm 54.2	707.1 \pm 96.3	422.3 \pm 51.8	425.0 \pm 58.3	434.2 \pm 95.0	434.2 \pm 95.0	424.9 \pm 54.2	434.2 \pm 95.0	425.0 \pm 58.3	434.2 \pm 95.0	434.2 \pm 95.0	434.2 \pm 95.0
Myrcene	985	26.3 \pm 2.6	34.3 \pm 2.8	28.6 \pm 1.7	32.2 \pm 2.6	41.7 \pm 10.4	24.6 \pm 2.4	26.2 \pm 3.5	41.7 \pm 10.4	24.6 \pm 2.4	28.9 \pm 4.3	22.0 \pm 4.0	22.0 \pm 4.0	26.2 \pm 3.5	22.0 \pm 4.0	28.9 \pm 4.3	22.0 \pm 4.0	22.0 \pm 4.0	22.0 \pm 4.0
(+)-3-Carene	1002	26.0 \pm 12.1	166.7 \pm 37.4	11.7 \pm 4.5	17.8 \pm 15.5	23.5 \pm 9.1	11.5 \pm 5.9	8.9 \pm 2.2	23.5 \pm 9.1	11.5 \pm 5.9	18.2 \pm 17.8	5.6 \pm 3.8	5.6 \pm 3.8	8.9 \pm 2.2	5.6 \pm 3.8	18.2 \pm 17.8	5.6 \pm 3.8	5.6 \pm 3.8	5.6 \pm 3.8
β -Phellandrene ¹	1022	88.6 \pm 9.9	104.4 \pm 10.9	99.9 \pm 8.1	99.6 \pm 11.4	152.4 \pm 30.9	76.8 \pm 8.8	84.8 \pm 19.1	152.4 \pm 30.9	76.8 \pm 8.8	88.0 \pm 15.7	71.3 \pm 12.3	71.3 \pm 12.3	84.8 \pm 19.1	71.3 \pm 12.3	88.0 \pm 15.7	71.3 \pm 12.3	71.3 \pm 12.3	71.3 \pm 12.3
Limonene ¹	1030	24.4 \pm 2.8	28.2 \pm 8.3	19.0 \pm 2.1	29.9 \pm 8.2	44.8 \pm 12.5	26.3 \pm 11.6	33.3 \pm 3.1	44.8 \pm 12.5	26.3 \pm 11.6	33.5 \pm 2.5	21.9 \pm 5.4	21.9 \pm 5.4	33.3 \pm 3.1	21.9 \pm 5.4	33.5 \pm 2.5	21.9 \pm 5.4	21.9 \pm 5.4	21.9 \pm 5.4
α -Terpinolene	1079	5.5 \pm 1.3	20.7 \pm 2.8	5.2 \pm 0.5	6.2 \pm 1.6	7.8 \pm 1.1	5.7 \pm 0.7	4.7 \pm 0.3	7.8 \pm 1.1	5.7 \pm 0.7	5.0 \pm 0.8	4.0 \pm 0.6	4.0 \pm 0.6	4.7 \pm 0.3	4.0 \pm 0.6	5.0 \pm 0.8	4.0 \pm 0.6	4.0 \pm 0.6	4.0 \pm 0.6
Bornyl acetate	1268	1.6 \pm 0.7 ^a	2.5 \pm 3.3	1.8 \pm 1.3	3.4 \pm 1.7 ^b	4.1 \pm 2.5	4.3 \pm 1.8	1.6 \pm 0.9 ^a	4.1 \pm 2.5	4.3 \pm 1.8	1.6 \pm 1.4	2.3 \pm 1.2	2.3 \pm 1.2	1.6 \pm 0.9 ^a	2.3 \pm 1.2	1.6 \pm 1.4	2.3 \pm 1.2	2.3 \pm 1.2	2.3 \pm 1.2
α -Longipinene	1347	5.1 \pm 1.5	3.5 \pm 0.8	3.1 \pm 0.6	6.6 \pm 0.8	7.0 \pm 0.4	3.4 \pm 0.2	4.7 \pm 2.7	7.0 \pm 0.4	3.4 \pm 0.2	4.9 \pm 1.4	2.9 \pm 1.4	2.9 \pm 1.4	4.7 \pm 2.7	2.9 \pm 1.4	4.9 \pm 1.4	2.9 \pm 1.4	2.9 \pm 1.4	2.9 \pm 1.4
Longifolene	1396	16.2 \pm 3.3	14.6 \pm 3.7	9.5 \pm 1.2	8.5 \pm 8.3	13.4 \pm 7.4	4.3 \pm 3.5	8.2 \pm 6.7	13.4 \pm 7.4	4.3 \pm 3.5	8.2 \pm 5.7	5.0 \pm 3.6	5.0 \pm 3.6	8.2 \pm 6.7	5.0 \pm 3.6	8.2 \pm 5.7	5.0 \pm 3.6	5.0 \pm 3.6	5.0 \pm 3.6
(E)- β -Caryophyllene	1411	12.2 \pm 6.8	13.2 \pm 6.7	9.1 \pm 3.7	5.7 \pm 3.6	7.3 \pm 4.1	3.8 \pm 2.5	10.5 \pm 2.0	7.3 \pm 4.1	3.8 \pm 2.5	8.9 \pm 2.5	6.9 \pm 1.9	6.9 \pm 1.9	10.5 \pm 2.0	6.9 \pm 1.9	8.9 \pm 2.5	6.9 \pm 1.9	6.9 \pm 1.9	6.9 \pm 1.9
(-)-Germacrene D	1473	15.2 \pm 15.2	13.6 \pm 14.1	9.2 \pm 5.9	31.8 \pm 15.2	36.5 \pm 19.8	10.6 \pm 6.5	13.6 \pm 12.4	36.5 \pm 19.8	10.6 \pm 6.5	10.3 \pm 8.2	8.1 \pm 7.1	8.1 \pm 7.1	13.6 \pm 12.4	8.1 \pm 7.1	10.3 \pm 8.2	8.1 \pm 7.1	8.1 \pm 7.1	8.1 \pm 7.1
δ -Cadinene	1506	9.1 \pm 1.0	6.4 \pm 1.8	3.0 \pm 1.3	12.6 \pm 3.5	12.3 \pm 8.0	3.0 \pm 1.7	11.7 \pm 8.9	12.3 \pm 8.0	3.0 \pm 1.7	9.1 \pm 6.9	3.6 \pm 2.2	3.6 \pm 2.2	11.7 \pm 8.9	3.6 \pm 2.2	9.1 \pm 6.9	3.6 \pm 2.2	3.6 \pm 2.2	3.6 \pm 2.2
Germacrene D-4-ol	1562	11.2 \pm 4.7	11.0 \pm 8.3	5.2 \pm 2.1	23.2 \pm 18.8	36.5 \pm 33.5	8.4 \pm 5.4	7.1 \pm 3.4	36.5 \pm 33.5	8.4 \pm 5.4	11.3 \pm 11.1	6.8 \pm 3.3	6.8 \pm 3.3	7.1 \pm 3.4	6.8 \pm 3.3	11.3 \pm 11.1	6.8 \pm 3.3	6.8 \pm 3.3	6.8 \pm 3.3
Thunbergol	2037	56.2 \pm 24.2	102.6 \pm 35.1	41.9 \pm 14.6	21.9 \pm 15.7	33.3 \pm 33.1	23.4 \pm 14.1	45.3 \pm 39.2	33.3 \pm 33.1	23.4 \pm 14.1	38.7 \pm 23.1	18.6 \pm 8.9	18.6 \pm 8.9	45.3 \pm 39.2	18.6 \pm 8.9	38.7 \pm 23.1	18.6 \pm 8.9	18.6 \pm 8.9	18.6 \pm 8.9
Neobienol	2123	98.1 \pm 23.2	104.6 \pm 24.3	94.7 \pm 6.9	102.0 \pm 17.1	151.3 \pm 16.5	91.6 \pm 7.9	91.4 \pm 38.1	151.3 \pm 16.5	91.6 \pm 7.9	107.5 \pm 10.2	93.9 \pm 11.9	93.9 \pm 11.9	91.4 \pm 38.1	93.9 \pm 11.9	107.5 \pm 10.2	93.9 \pm 11.9	93.9 \pm 11.9	93.9 \pm 11.9
Abienol	2126	48.5 \pm 4.9	43.6 \pm 10.7	40.0 \pm 2.7	56.0 \pm 6.3	61.5 \pm 6.1	38.1 \pm 2.6	57.9 \pm 11.8	61.5 \pm 6.1	38.1 \pm 2.6	49.1 \pm 4.9	36.2 \pm 4.5	36.2 \pm 4.5	57.9 \pm 11.8	36.2 \pm 4.5	49.1 \pm 4.9	36.2 \pm 4.5	36.2 \pm 4.5	36.2 \pm 4.5
Total		1500.8 \pm 120.5	1789.4 \pm 143.3	1545.9 \pm 89.5	1549.2 \pm 86.7	2070.2 \pm 216.5	1327.6 \pm 57.3	1517.3 \pm 165.7	2070.2 \pm 216.5	1327.6 \pm 57.3	1452.3 \pm 131.3	1338.8 \pm 127.6	1338.8 \pm 127.6	1517.3 \pm 165.7	1338.8 \pm 127.6	1452.3 \pm 131.3	1338.8 \pm 127.6	1338.8 \pm 127.6	1338.8 \pm 127.6

¹ For absolute amounts of (+) and (-) enantiomers of α -pinene and limonene, and (-)-enantiomers of β -pinene and β -phellandrene, see Fig. 2

² Kovats retention index

Boldface numbers indicate significant differences between day 0 and day 30 within treatments; different letters indicate significant differences between treatments on day 0 ($p < 0.05$).

Table 2. Terpene concentration ($\mu\text{g g}^{-1}$ dry wt equivalent to pentadecane) in stem phloem of putative susceptible Norway spruce trees with long lesions (> 50 mm, $n = 3$) and putative resistant trees with short lesions (< 30 mm, $n = 7$) following inoculation with *Ceratocystis polonica*. Terpene concentrations were measured one month after inoculation and 5 cm above the inoculation site. Data are expressed as means \pm 1 SD and compared by t-test.

	Lesion length < 30 mm	Lesion length > 50 mm	<i>p</i>
Total monoterpenes	1396.2 \pm 24.0	1321.7 \pm 71.6	0.907
Total sesquiterpenes	93.1 \pm 24.0	70.0 \pm 13.2	0.693
Total diterpenes	412.4 \pm 27.7	133.1 \pm 5.8	0.023
Thunbergol	175.5 \pm 22.7	14.3 \pm 5.4	0.011

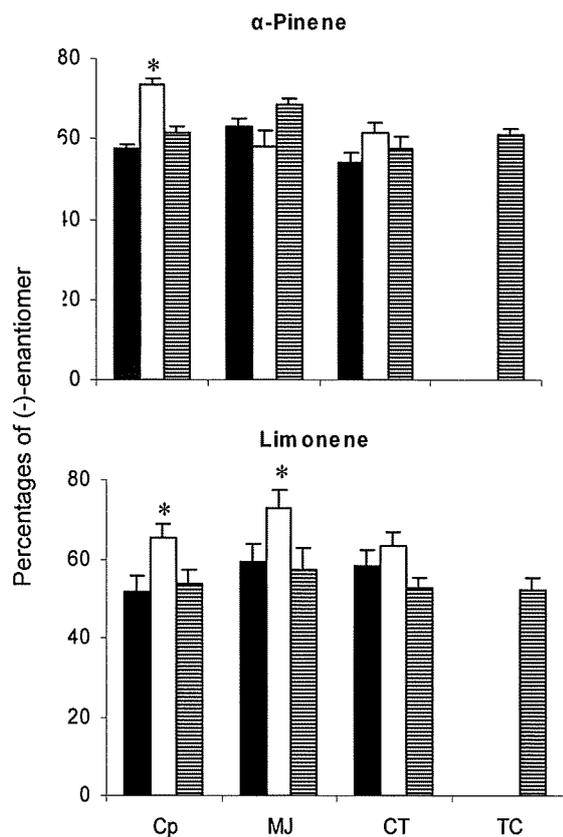


Fig. 1. Enantiomeric composition (mean + 1 SD) of α -pinene and limonene before treatment with methyl jasmonate (MJ) or *Ceratocystis polonica* (Cp) (black bars) and one month after treatment (white bars: treated stem section; hatched bars: untreated stem section). CT denotes untreated control trees that were also sampled at the start of the experiment; TC is untreated control trees with no previous sampling history. Data are expressed as the percentage of the (-)-enantiomer to the sum of both enantiomers. $n = 8$ trees (except for TC where $n = 5$). No difference in enantiomeric composition was observed before treatment or in the untreated stem section after treatment between trees assigned to different treatments. Stars indicate enantiomeric composition significantly changed in treated stem section after treatment by t-test ($p < 0.05$).

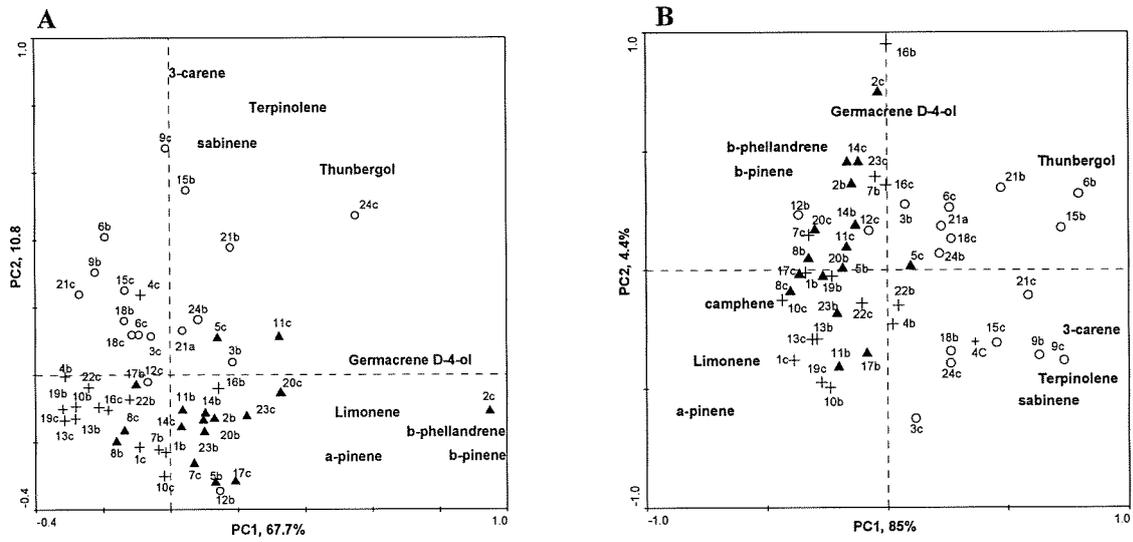


Fig. 2. PCA plots for absolute amounts (A) and relative proportions normalized to 100% (B) of all the quantified terpenes in treated Norway spruce phloem 30 days after stem treatment with *Ceratocystis polonica* (circles) or methyl jasmonate (triangles). Crosses represent untreated control trees. Each symbol represents one tree. Tree no. is indicated next to the symbols. The terpene position indicates its approximately contribution to the principal component. In panel A the first principal component (PC1) explained 67.7%, and the second component (PC2) 10.8% of the sample variation. In B, PC1 explained 85.0%, and PC2 explained 4.4% of the sample variation.

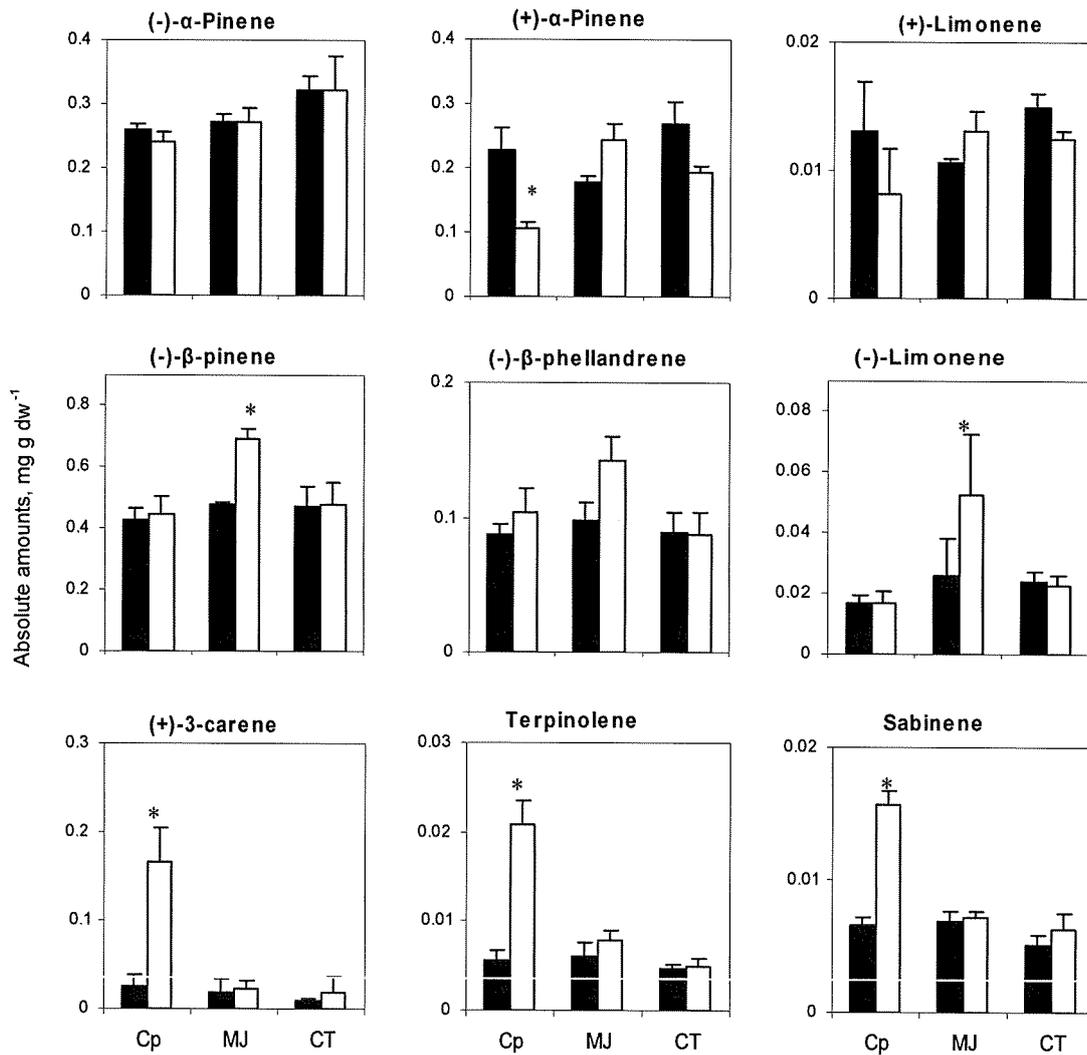


Fig. 3. Absolute amounts of selected monoterpenes in Norway spruce phloem before (day 0, black bars) and 30 days after (white bars) stem treatment with methyl jasmonate (MJ) or inoculation with the bluestain fungus *Ceratocystis polonica* (Cp). CT denotes untreated control trees. Data are from the treated section of the stem and is expressed as means + 1 SD. n = 8 trees, except for the three lower panels where n = 16 trees. Bars with stars were significantly different by t-test ($p < 0.05$).

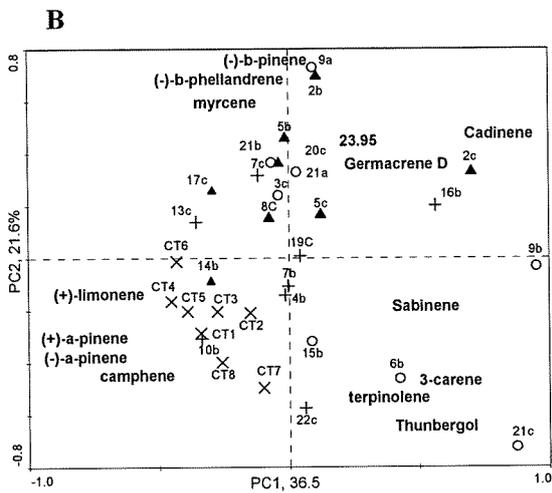
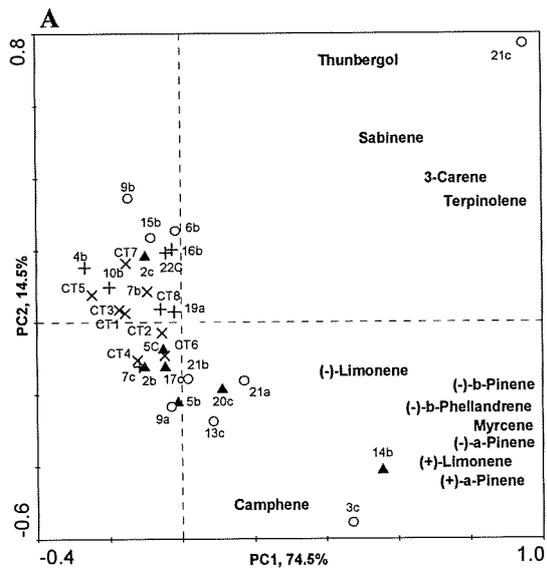


Fig. 4. PCA plots based on absolute (A) and relative amounts normalized to 100% (B) of all the quantified terpenes in Norway spruce phloem 362 days after stem treatment with *Ceratocystis polonica* (circles) or methyl jasmonate (triangles). + = untreated control trees that were also sampled at the start of the experiment; × = untreated control trees with no previous sampling history. Each symbol represents one tree. Tree no. is indicated next to the symbol. The terpene position indicates its approximately contribution to the principal components. In panel A the first principal component (PC1) explained 74.5%, and the second component (PC2) explained 14.5% of the sample variation. In B, PC1 explained 36.5%, and PC2 explained 21.6% of the sample variation.

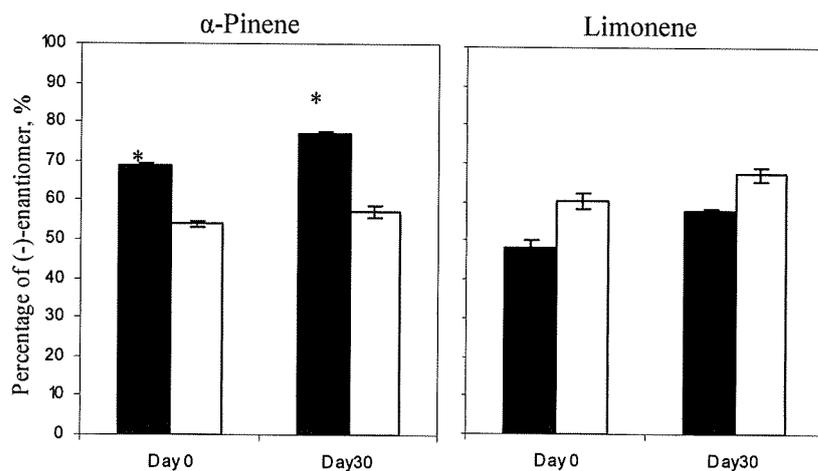


Fig. 5. Enantiomeric composition of α -pinene and limonene in six relatively attractive (black bars) and seven relatively unattractive Norway spruce trees (white bars). Attractive trees had > 20 spruce bark beetles landing on them and/or > 10 beetles entering; unattractive trees had < 5 beetles landing or entering. Data are expressed as mean \pm 1SD. Bars with stars were significantly different by t-test ($p < 0.05$).

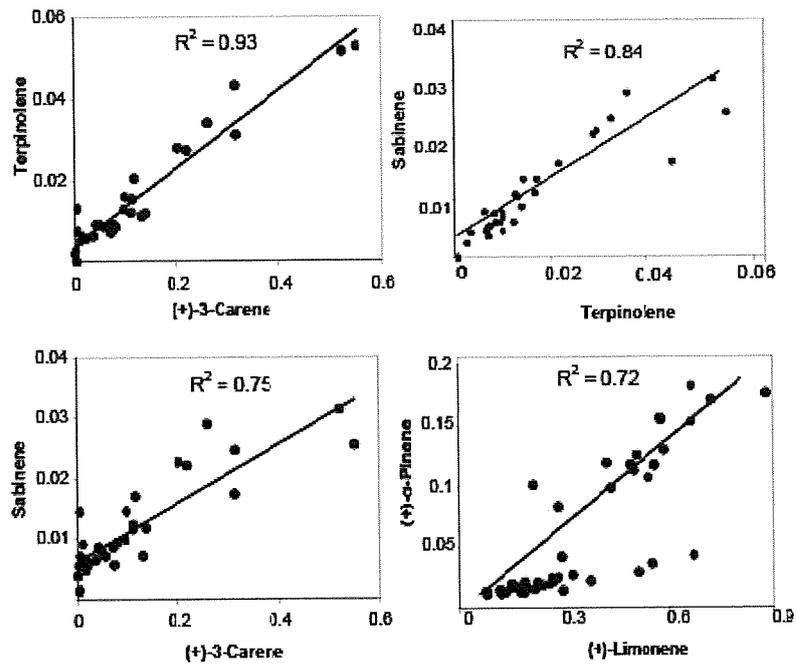


Fig. 6. Correlation between absolute amounts (mg g^{-1} dry wt equivalent to pentadecane) of different monoterpenes in Norway spruce. Data includes all the analyzed trees before treatment and 30 days after treatment ($n = 32$ for (+)- β -carene, sabinene and terpinolene; $n = 48$ for (+)- α -pinene and (+)-limonene).