Stress Related Emissions of Norway Spruce Plants

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Abstract

The interactions between plants and insects are mediated by volatile molecules. Plants respond to stress by biosynthesis of chemical substances which can deter invading insects or pathogens. Some of these substances are volatile and are emitted to the surroundings and may attract or repel insects. Information about the susceptibility of individual plants to infestation, their volatile emissions and chemical defence is of interest, for example in selecting plants for tree breeding programs.

This research was focused on finding volatile chemical markers of resistance in Norway spruce plants that do influence insects associated to conifers. Collection of headspace volatiles by SPME followed by separation and identification with GC-MS is effective in investigating biological systems with a minimum of disturbance. This method has here been used to investigate Norway spruce plants of different ages and stress conditions as well as trapping semiochemicals like nepetalactone emitted by the spruce shoot aphids. It was even possible to analyse the emission of single needles in vivo and obtain a chemical pattern of the site of the stress reaction. Seedlings of different ages showed differences in chemical composition of emitted volatiles, with the pine weevil repellent (S)-(-)-limonene as one of the main compounds. Wounded phloem of conventional plants emitted high amounts of monoterpenes while the phloem of mini plants emitted (3Z)-hexenal and (3Z)-hexen-1-ol.

Norway spruce plants did respond to different stress elicitors with similar response, regardless of their genetic origin. The emissions from stressed Norway spruce plants mainly consist of (E)-β-farnesene, (E,E)-α-farnesene, (E)-α-bisabolene, (R)-(-)-linalool and methyl salicylate. Emissions from live spruce shoot aphids were detected during autumn periods, and a method to separate and identify the four diastereomers of nepetalactone by GC-MS and characteristic m/z-fragments was accomplished.

Key words: Picea abies, Hylobius abietis, Cinara pillicornis, volatiles, terpenes, green leaf volatiles, stress response, nepetalactone, SPME.
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I. Pine weevil damage on planted spruce seedlings may be explained by plant chemical signals.

II. Single needle analyses by SPME-GC-MS, a miniaturized method to localize induced stress sites in conifers.
    Pettersson M. and Borg-Karlson A.-K. Manuscript

III. Semiochemicals related to the aphid Cinara pilicornis (Hartig) and its host, Picea abies. A method to assign diastereomers of nepetalaactone.
    Pettersson M., Unelius R. and Borg-Karlson A.-K. Manuscript

Abbreviations

ec           Enantiomeric composition
GC           Gas chromatography
GLV          Green leaf volatiles
LOX          Lipoxygenase
MeJA         Methyl jasmonate
MEP          Methyl-D-erythriol-1-phosphate
MeSA         Methyl salicylate
MS           Mass spectrometry
MT           Monoterpene
MT-O         Oxygenated monoterpene
MVDA         Multivariate data analysis
PCA          Principal component analysis
PP cell      Polyphenolic parenchyma cell
RDA          Redundancy analysis
SPME         Solid phase microextraction
SqT          Sesquiterpene
TD           Traumatic resin duct
TPS          Terpene synthase
Introduction

Aim

The aim of my research is to find chemical markers of resistance in conifers. This thesis focuses on volatile chemical resistant markers produced by plants of Norway spruce of different ages, both during non stressed and stressed development.

Background

Insects use volatiles as their main way of communication; they use their sense of smell to find food, a partner or an appropriate place to lay their eggs. For plants, which cannot escape threats like herbivore insects, chemical constituents play an important role in their own defence system. One of a few ways for plants to defend themselves is to send volatile compounds as signals to the world surrounding them. Molecules that transfer information between organisms are called semiochemicals and the study of interactions in nature mediated by semiochemicals is called chemical ecology.

Conifers and their defence strategies

Conifers are long lived trees belonging to the gymnosperms, which contain many species that successfully inhabit large areas of our planet. As raw material for many products (wood, paper, plastics, fuel, chemicals, etc) their economic impact on our society is of great importance. In Sweden, 83 % of the forests consist of conifers, mainly Norway spruce (Picea abies) and Scots pine (Pinus sylvestris) (Alin and Sundberg, 2003). Conifers produce huge amounts of compounds, terpenes, displaying large variations in composition between individual trees of the same species (Persson et al., 1996), which is one of the reasons for their successful survival on earth. The variations in chemical composition lead to differences in susceptibility to insects and pathogens, and the chemical markers of resistance can be constitutive compounds as well as induced ones (Brignolas et al., 1998, Franceschi et al., 2005, Almquist et al., 2006).

Terpenes and phenolics are continuously produced by the plants of the family Pinaceae and are stored in special structures, resin ducts, within the tree (Franceschi et al., 2005). The constitutive terpenes function as a defence against invading insects. At mechanical wounding the resin ducts get punctured and resin flows out, deters the insect and seals the wound (Phillips and Croteau, 1999). In addition, conifers have an induced chemical defence; biotic, abiotic and synthetic stress elicitors activate biosynthetic pathways (Franceschi et al., 2005, Keeling and Bohlmann, 2006, Phillips et al., 2006). The induced defence leads to the formation of new traumatic resin ducts and polyphenolic parenchyma cells (Franceschi et al., 2002, Franceschi et al., 2005) as well as altered quantities and compositions of both terpenes (Sjödin et al., 1993, Fäldt et al., 2006, Phillips et al., 2006) and phenolics (Evensen et al., 2000, Viiri et al., 2001, Franceschi et al., 2005). The stress-induced changes do not only occur within the trees. Conifers release volatiles (mainly terpenes) from their needles and the amount and quality of the emitted terpenes change upon stress (Martin et al., 2003, Miller et al., 2005).
Biosynthesis and function of conifer volatiles

Terpenes are produced through two different biosynthetic pathways. Sesquiterpenes are formed through the melvanoate pathway in the cytosol, monoterpenes and diterpenes via the methyl-D-erythriol-1-phosphate (MEP) pathway in the plastids (Figure 1). Both pathways give rise to the substrates of all terpenes: isopentenyl diphosphate and dimethylallyl diphosphate and exchanges of these compounds occur between the cytosol and the plastid (Rohmer, 1999, Dudareva et al., 2004). They condense and form the precursors of monoterpenes (geranyl diphosphate), sesquiterpenes (farnesyl diphoshpate) and diterpenes (geranylgeranyl diphosphate) which are then transformed by terpene synthases to the various terpene products of the plant. In the last few years a lot of work has been devoted to identifying and characterizing the terpene synthases in conifers. So far about 40 terpene synthases have been characterized in 13 different conifer species (for a recent review see Keeling and Bohlmann 2006), of these 10 in *Picea abies* (Fäldt et al., 2003, Martin et al., 2004). Some terpene synthases give rise to highly specific products (e.g. Bohlmann et al., 1998) while others produce a mixture of multitude products (e.g. Steele et al., 1998). A number of terpene synthase transcripts are up-regulated upon stress leading to induced biosynthesis of terpenes (Huber et al., 2004, Keeling and Bohlmann, 2006).

**Figure 1.** Biosynthesis of terpenes occurs through two different biosynthetic pathways. DMAPP: dimethylallyl diphosphate, FPS: farnesyl diphosphate synthase, GGPS: geranylgeranyl diphosphate synthase, GPS: geranyl diphosphate synthase, IPP: isopentenyl diphosphate, MEP: methyl-D-erythritol-1-phosphate and TPS: terpene synthase.

Metabolites of the lipoxygenase (LOX) pathway are also highly associated with stress signalling in plants. Lipids from the plant membranes are cleaved and transformed into jasmonates and green leaf volatiles through different branches of the LOX pathway (Figure 2; Blee, 1998). Green leaf volatiles (GLVs) constitute of C6 aldehydes, alcohols and esters which give the characteristic smell of freshly cut grass (Hatanaka, 1993) and are common in deciduous trees and plants. GLVs are rapidly
formed upon mechanical damage of green leaves, probably because of the mixture of membrane lipids with enzymes liberating the fatty acids, thus supplying the substrates for the biosynthesis (Paré and Tumlinson, 1999, Matsui, 2006). But the compounds are also produced upon stress without mechanical damage as well as systemically from other parts of the plant; this demonstrates that mechanical damage is not necessary for production (Matsui, 2006).

**Figure 2.** Part of the lipoxygenase pathway. LOX: lipoxygenase, AOS: allene oxide synthase, JMT: jasmonic acid methyl transferase, HPL: hydroperoxide lyase and ADH: alcohol dehydrogenase. Jasmonates, green leaf volatiles and traumatic acid are all compounds associated to stress in plants.

Among the main volatiles produced by conifers upon stress are the farnesene sesquiterpenes (Figure 3) and the monoterpen linalool (Martin *et al.*, 2003, Mumm *et al.*, 2003, Miller *et al.*, 2005). These compounds are also associated with stress-induced volatiles of a number of angiosperms (Paré and Tumlinson, 1999). Therefore, the farnesenes are suitable target compounds to monitor volatile stress reactions in plants.

**Figure 3.** There exist six different farnesene isomers, (E)-β-farnesene and (E,E)-α-farnesene are common in the volatile headspace of stressed plants.

**Insects**

The odour change in host plants can affect the interactions with insects; the attraction to the plant can be altered and/or the induced volatiles can attract predators to the herbivores (Arimura *et al.*, 2005, Dudareva *et al.*, 2006). There are a number of
insects and other arthropods associated to conifers; the ones that cause economical damage are defined as pests (Berryman, 1982). The organisms themselves may be the main cause of damage to the plant, but they can also be vectors for fungal pathogens and other diseases. The conifer associated arthropods use different strategies to colonize and feed on the plants.

The large pine weevils, *Hylobius abietis* (L.), feed from the bark and phloem of the plants and are a great problem to the Swedish forestry since they damage and kill newly planted seedlings of pine and spruce (Långström and Day, 2004). If pesticides had not been used, the estimated costs would have been 400 million SEK higher every year (Thuresson *et al.*, 2003). The weevils fly to the clear-cuttings guided by volatiles emitted from the freshly cut stumps where they lay their eggs and feed on the newly planted seedlings.

The spruce shoot aphid, *Cinara pilicornis* (Hartig) also feed from the phloem of Norway spruce but use another feeding strategy. Aphids penetrate the bark with their stylets and get nourishment from the sap. They locate their hosts both through volatiles as well as through chemical cues found when penetrating the plant (Powell and Hardie, 2001). Other organisms such as the conifer spinning mite (Tetranychidae) or the mite *Nalepella haarlovi* Boczek var. *Picea abietis* Löyttyniemi (Acarina, Ereophyidae) live and feed on the conifer’s needles (Kännaste *et al.*, submitted).

This study

Emissions of spruce plants of different ages and conditions have been investigated in order to elucidate possible volatile markers of resistance in Norway spruce that may influence the behaviour of arthropodes (attractants or repellents).

1. Mini seedlings (6-10 weeks old) were compared with conventional seedlings (1-2 year old) to look for compounds explaining the different susceptibility to the large pine weevil (I). Analyses were made of volatiles from:
   a. Intact plants
   b. The phloem after wounding

2. The volatile reaction of Norway spruce plants to different stress elicitors were analysed and compared:
   a. Methyl jasmonate (II)
   b. Spruce shoot aphid (III)

3. The solid phase microextraction technique was adjusted to measure volatiles from single needles *in vivo*. This method was used to locate the volatile stress response to the chemical stress elicitor methyl jasmonate as well as to mechanical wounding (II).

4. Semiochemicals and possible pheromonal compounds were identified in the headspace around the spruce shoot aphids and a method to elucidate the diastereomers of nepetalactone with gas chromatography – mass spectrometry (GC-MS) was developed (III).
Materials and methods

Biological material

The studies were carried out on Norway spruce (Picea abies (L.) Karst.) plants of various ages. The young seedlings referred to as mini seedlings were six to ten weeks old, while the conventional seedlings, normally used for reforestation, were between one and two years old. Clones show less variation in terpene content than seedlings do, due to their identical genetic origin (Hanover, 1992, Silvestrini et al., 2004). This makes them suitable to use in comparative studies to minimize variation between plants. The clones used in manuscript I and II are two to three year old cuttings from archives of Skogforsk (the Forestry Research Institute of Sweden) with known resistance to the large pine weevil. Spruce shoot aphids (Cinara pilicornis) were obtained from naturally infested plants.

Collection and analysis of volatiles

Volatile are mediators in the first step of interaction (i.e. host location) between plants and insects. There are no observed correlations between the chemical content in the foliage of a plant and the compounds emitted to its environment (Schindler and Kotzias, 1989, Martin et al., 2003). To decipher the signals a plant sends out it is therefore necessary to collect and analyse the compounds in its headspace, i.e. the air above and around the plant. The most common way to separate and identify the contents of volatile samples is the use of gas chromatography coupled to mass spectrometry (GC-MS). This is the technique, selected in this work together with the two-dimensional gas chromatographic system described by Borg-Karlson et al., (1993), used to separate the enantiomers of monoterpenes.

Solid phase microextraction (SPME)

Several methods to collect headspace volatiles are used in ecological chemistry (Agelopoulos and Pickett, 1998, Millar and Sims, 1998, D’Alessandro and Turlings, 2006) whereof solid phase microextraction (SPME) is one. SPME is a simple, fast, non-destructive, solvent free extraction technique. Originally, this it was developed for analysis of organic pollutants in water samples, but has gained extensive use for sampling volatile compounds in headspace (Zhang and Pawliszyn, 1993, Pawliszyn, 1999, Augusto and Valente, 2002). It has successfully been used in the analysis of headspace volatiles from both plants (I; II; III; Schäfer et al., 1995, Augusto and Valente, 2002, Zini et al., 2002) and insects (III; Borg-Karlson and Mozuraitis, 1996, Moneti et al., 1999, Andersson et al., 2000, Augusto and Valente, 2002, Andersson et al., 2007).

The SPME device consists of a silica fibre with a polymer coating mounted on a syringe-like holder. Analytes are extracted from the headspace and concentrated on the fibre. After exposure to the sample the fibre is retracted into the needle and injected into a GC-MS desorbing the analytes for further identification.

SPME is a selective method and there are several fibre coatings available to facilitate extraction of diverse compound classes. The mixed coating polydimethylsiloxane / divinylbenzene (PDMS/DVB) is suitable for volatile compounds (C6-C15) such as terpenes emitted by conifers (Mani, 1999, Fäldt et al., 2000). The compounds are extracted through adsorption rather than absorption and possible
competition effects should be considered (Gorecki et al., 1999). To avoid this, short extraction times are recommended for porous fibres (Pawliszyn, 2002) but when studying plant emissions there are additional factors to consider concerning the sorption time. The volatiles emitted by plants vary, as previously mentioned, by genetic origin, but temperature, light conditions, season and time of day also affect the emissions (Kesselmeier and Staudt, 1999, Staudt et al., 2000, Niinemets et al., 2002, Niinemets et al., 2004, Hakola et al., 2006). To be able to compare the odour bouquets between the plants both biotic and abiotic factors must be taken into account and be similar for all analyses. Small amounts (ng) are continuously emitted by the plants, thus long extraction times are needed. When long adsorption times (20-24 h) are used, the enclosed headspace of the plant will consist of the volatiles produced during the whole day and possible diurnal effects will not be recognized. The long extraction times contradict the general recommendations set by measuring static headspace samples. However, long extraction time enhance the proportions of larger molecules collected by the fibre, e.g. (E)-β-farnesene, one of the key compounds in stress reactions of conifers.

Possible competition effects of other terpenes or water on the adsorption of (E)-β-farnesene were investigated in manuscript II. No significant differences in adsorbed amounts could be detected with the long sorption times, neither for the compound in a terpene mixture with all compounds of equal concentration, nor for (E)-β-farnesene in the terpene mixture with a large excess of water (Figure 4). Water is emitted by the plants during respiration and was often found condensed on the inside of collection jars after long adsorption times. Two compounds were collected in significantly less amounts when water was present; α-pinene (p = 0.005) and limonene (p = 0.017, two-tail p values from t-test assuming unequal variance).

![Chromatogram areas](image)

**Figure 4.** Chromatogram areas of seven compounds typical in the headspace of spruce collected with SPME (PDMS/DVB) in the single needle setup (II). 1) Terpene mixture of equal concentrations (5 replicates). 2) The same terpene mixture with added water, 100 000 times the terpene concentration (7 replicates). Error bars denote standard deviations.

**Statistical analysis of GC-MS data**

When a few variables are analysed it is convenient to describe the data by means and standard deviations, and to analyse significant differences between samples with t-tests or one way ANOVA. However, in plant chemistry it is often necessary to deal with many variables. Conifer plants can emit up to one hundred different compounds and it is not necessarily the most abundant ones that cause the largest ecological
impact. To group plants based on the volatiles emitted, all the compounds need to be considered and for this purpose multivariate data analysis (MVDA) is useful (Wold et al., 1989, Persson et al., 1996, Silvestrini et al., 2004).

Principal component analysis (PCA) is an ordination method that places the samples in an n-dimensional space where n equals the number of descriptive variables (e.g. chemical compounds). The samples are then projected onto a two-dimensional plane retaining as much of the variation between the samples as possible. The axes of the new plane are called principal components (PCs) and are presented together with a percentage describing how much of the variation in the data set is explained by the PC. The descriptive variables may be projected on the new plane either as vectors or as marks and be plotted in a separate loading plot or in a biplot together with the samples. The impact of a variable to the PCs increases with its distance to origo. Samples placed close to each other on the score plot have similar variable properties; in this thesis that will typically mean similar chemical composition. The placements of variables close to each other indicate the occurrence of covariation. For a tutorial on PCA see Wold et al. (1987).

Redundancy analysis (RDA) is a constrained ordination method (Rao, 1973). It is an extension of PCA where a second set of explanatory variables are included. For example explanatory variables may consist of information about plant resistance or nature of stress elicitor. The ordination axes of RDA are thus constructed to separate the samples according to the explanatory variables in a way to explain as much as possible of the variation in the data set (Økland, 1996). RDA can be used to find out which descriptive variables differentiate predefined groups of samples, e.g. to find pollution sources giving rise to specific chemical responses in lichen (Gonzalez et al., 2003).

The Data Analysis tool in Excel was used for t-tests and one way ANOVA and the statistical analysis software CANOCO (Version 4.54, developed by Cajo J. F. Ter Braak and Petr Smilauer, Biometris Plant Research International, The Netherlands) for PCA and RDA.

**Headspace analysis of single needles in vivo (II)**

To monitor the volatile stress reactions in spruce we developed a system to collect the volatiles of single needles still attached to the plant. A thin glass tube (2-3 mm in diameter, 8 cm in length) was placed over the needle, a SPME were placed into the glass tube and the fibre was exposed during 22 hours, collecting the volatiles from the needle. As background check a similar glass tube with SPME was mounted against the stem of the plant.

The single needle setup made it possible to follow the change in volatile emission upon stress from one separate needle. The setup was used to monitor how far the stress signalling was transported in the stem, from where the synthetic stress elicitor methyl jasmonate was applied, as well as from the site where the stem was mechanically wounded (described under “Localization of the volatile response”). Clear differences could be found in the emission from needles growing at the site of stress and at a short distance away from it, implying a local volatile reaction.
Identification of nepetalactone diastereomers with GC-MS and multivariate analysis (III)

To assign the correct isomer to a compound analysed by GC-MS is not always straightforward since many stereoisomers have very similar mass spectra. However, small differences often occur and by using multivariate analysis these can be found (Le Bizec et al., 2005, Berman et al., 2006). We used MVDA to find characteristic fragments of the four diastereomers of nepetalactone (III), of which the cis-trans one has been characterized as a component of sex pheromones in several aphid species (Dawson et al., 1990, Birkett and Pickett, 2003). cis-trans-Nepetalactone was found together with cis-trans-nepetalactol and citronellol in the headspace of the spruce shoot aphid during short periods in autumn for two succeeding years. During the structure identification a simple test was developed to assign the correct diastereomer to an unknown nepetalactone.

Studies on one GC-MS instrument revealed clear impact of concentration on MS-spectra even after the intensities of the m/z-peaks in the spectra had been normalized (maximum peak in each spectra was set to 100%). There was a tendency of the samples to be grouped according to isomer identity but concentration differences were too large to allow clear grouping. A constrained ordination, RDA, revealed m/z fragments which could be useful to separate the nepetalactone isomers. From the RDA analysis the m/z-fragments with highest impact on the principal component axis were chosen: 85, 111, 137, 138 and 151. Using this selection of m/z fragments in PCA gave a plot with four groups corresponding to the four diastereomers (Figure 5).

Figure 5. PCA of nepetalactone isomers based on relative abundances of five selected m/z-fragments (denoted by vectors in the plot). Symbols: ◊: trans-trans-nepetalactone, □: cis-trans-nepetalactone, △: trans-cis-nepetalactone, ×: cis-cis-nepetalactone and +: nepetalactone from aphids. The aphid nepetalactones were not included in the ordination but plotted into the PCA afterwards. Each nepetalactone isomer has been encircled in the plot.
A simpler test was developed based on these m/z fragments and retention indices (Table 1) which works well also with other quadrupol instruments (III). To test the method, the natural nepetalactone samples collected from the headspace of the spruce shoot aphid (*Cinara plicicornis*) were used. The test unambiguously assigned all the replicates (n = 10) of the aphid nepetalactone to be *cis-trans*-nepetalactone, and when plotted into the PCA in Figure 5 all aphid samples belonged to this nepetalactone group. This identification could be confirmed by injection on two columns and comparing retention indices with standards (III).

**Table 1.** Test to assign nepetalactone isomers by comparing characteristic fragments in MS-spectra.

<table>
<thead>
<tr>
<th>Nepetalactone isomers</th>
<th><em>trans-trans</em></th>
<th><em>cis-trans</em></th>
<th><em>trans-cis</em></th>
<th><em>cis-cis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>85 &lt; 137</td>
<td>85 &gt; 137</td>
<td>85 &lt; 137</td>
<td>85 &gt; 137</td>
<td></td>
</tr>
<tr>
<td>110 &gt; 111</td>
<td>110 &gt; 111</td>
<td>110 &gt; 111</td>
<td>110 &lt; 111</td>
<td></td>
</tr>
<tr>
<td>137 &gt; 138</td>
<td>137 &lt; 138</td>
<td>137 &lt; 138</td>
<td>137 &gt; 138</td>
<td></td>
</tr>
<tr>
<td>138 &lt; 151</td>
<td>138 &gt; 151</td>
<td>138 &gt; 151</td>
<td>(138 ≈ 151)</td>
<td></td>
</tr>
</tbody>
</table>
Conifer reforestation is normally performed with one to two years old seedlings of spruce or pine. Currently, a reforestation system with so called mini seedlings (6-10 weeks old plants) is under evaluation in Sweden (Gyldberg and Lindström, 1999) and the trials show that the mini seedlings get less attacked by the large pine weevil (*Hylobius abietis*) than the conventional seedlings do (I). The mini seedlings are discovered by the weevils to the same extent as the conventional ones are (Mitsell, 2005), but they do not trigger the same feeding behaviour upon discovery. This may be explained by differences in the volatiles emitted by the plants, since the large pine weevils partly orient themselves by odours (Björklund *et al.*, 2005).

The large pine weevils have several antennal odour receptors which have been shown to respond selectively to a large number of compounds (Mustaparta, 1975). The pine weevils have one receptor type which is selectively tuned more to the (+)-enantiomer of α-pinene and another receptor type responds more to (S)-(−)-limonene than to their enantiomeric counterparts (Figure 6; Wibe and Mustaparta, 1996, Wibe *et al.*, 1998). (S)-(−)-Limonene is a known repellent to the large pine weevil (Nordlander, 1990), and both enantiomers of α-pinene are attractants. Therefore, the enantiomeric composition of these compounds in the emissions of the seedlings may affect the weevils and is thus of importance to measure.

![Figure 6. Structures of α-pinene and limonene. The enantiomers are perceived differently by the large pine weevil.](image)

The volatiles of the plants were studied to explain the difference in attack, by answering the following questions:

1. Do conventional seedlings emit higher amounts of volatiles than the mini seedlings?
2. Do the volatile compositions differ due to age between the two plant types?
3. Does the composition of compounds released by the phloem upon wounding differ between the plant types?
4. Does the enantiomeric composition of limonene and α-pinene differ between the volatiles of unwounded plants and volatiles released from phloem upon wounding? And does it differ between plants of different age?
Differences in volatiles from unwounded mini seedlings and conventional seedlings

It could be expected that the small mini seedlings with fewer needles would emit a smaller amount of volatiles to the surrounding air than the larger conventional seedlings. However, results from seedlings placed in containers of the same size were not unambiguous (Figure 7). Even though the average emissions from mini seedlings were lower than the average emission from conventional seedlings, the differences between the two seedling ages were not conclusive. This was due to the larger variation in the emissions from the older seedlings. Some conventional seedlings emitted up to three or even seven times more terpenes than the mini seedlings, but there were also seedlings emitting the same amounts as mini seedlings, despite the much larger size and needle mass of the older plants.

Not only the amounts of emitted compounds differed between the plants, the quality of the volatile blend varied as well. The compounds emitted by the spruce plants were divided into four groups according to their biosynthetic pathways; green leaf volatiles (GLV), monoterpenes (MT), oxygenated monoterpenes (MT-O) and sesquiterpenes (SqT). The last group also included oxygenated sesquiterpenes. The volatiles of the conventional seedlings consisted mainly of sesquiterpenes, which in average constituted 75% of the blend (Figure 8). Sesquiterpenes did not play such a prominent role in the mini seedling emissions, instead a more even distribution of compounds between the three terpene classes was observed.

Dividing the compounds into groups gives an indication about differences in the biosynthesis and emission between the plant age groups. But present knowledge of the odour perception and preferences of the pine weevil has also to be considered to find explanations to the lower susceptibility of mini seedlings as compared to the large pine weevils. (S)-(−)-Limonene, a repellent of the large pine weevil, was the major compound emitted by the unwounded mini seedlings and constituted in average 16 % of the volatile blend. Its enantiomeric counterpart (R)-(−)-limonene was also present, 4 % of the total amount of terpenes emitted (er\textsuperscript{1} = 80:20, S:R). These compounds were also present in the blend of conventional seedlings, (S)-(−)-limonene making out 4 %

\textsuperscript{1} Enantiomeric ration (ec) is “the ratio of the percentage of one enantiomer in a mixture of that of the other”, according to the definition of IUPAC (1996).
and (R)-(+) -limonene 2 % of the volatiles emitted (er = 67:33). The attractive α-pinenes could not be detected at all or were only present in trace amounts in the volatiles from the mini seedlings. In the headspace of conventional seedlings the two α-pinenes were present (in average 1.2 % of the compounds emitted) and the enantiomeric composition could be determined for four of the plants (minimum amount for enantiomeric analyzes was 1 ng), the composition was in average 65% (1S,5S)-(−)-α-pinene and 35% (1R,5R)-(+) -α-pinene.

Figure 8. Relative amounts of volatiles released by unwounded mini seedlings and conventional seedlings. GLV: green leaf volatiles, MT: monoterpenes, MT-O: oxygenated monoterpenes and SqT: sesquiterpenes. No GLV’s were observed in the headspace of unwounded seedlings. The emissions of each plant type were normalized to 100% individually, error bars denote standard errors.

Differences in volatiles from wounded mini seedlings and conventional seedlings

The large pine weevils are more attracted to wounded spruce plants than to unwounded ones (Tilles et al., 1986, Nordlander, 1991). Upon mechanical damage of the phloem the compounds, mainly terpenes, stored in compartments of the tissue are released into the air. These might differ from the ones emitted through the needles of unwounded plants (Miller et al., 2005).

In order to investigate the change in terpene emission upon wounding two strategies were used. First, to see if wounding would change the enantiomer composition of limonene and α-pinene the phloem of the seedlings, previously analysed in unwounded conditions were pierced with a needle and the volatiles were collected with SPME. Since large amounts were emitted, the volatiles were only sampled during a ten minute period, which means that the volatiles collected were mainly the terpenes passively emitted from the fresh wound. Secondly, the composition of the volatiles emitted from the wound was analysed; for this a new set of seedlings and a simplified setup was used. A small piece of phloem was removed from the stem base of the plants and placed in a small (3.5 ml) vial, the volatiles were thereafter collected with SPME for a 15-minute period and analysed with GC-MS.

As can be seen in Figure 9, the relative compositions of the phloem emissions from the two seedling ages differed significantly (Monte Carlo test, p = 0.0001). The phloems from mini seedlings only emitted small amounts. Their emissions mainly consisted of two compounds, the green leaf volatiles (3Z)-hexen-1-ol and (3Z)-hexenal (Figure 10), together making out in average 86% of the volatile blend. The phloems of the conventional plants, on the other hand, had a large emission, both
in amount emitted and number of compounds. The main compound class emitted was monoterpenes, of these the two enantiomers of α-pinene made up 38% and the ones of limonene 3%.

![Figure 9. Relative amounts of volatiles emitted by detached phloem pieces. There was a significant difference in composition of the emission from the phloems of the two plant types. GLV: green leaf volatiles, MT: monoterpenes, MT-O: oxygenated monoterpenes and SqT: sesquiterpenes. The emissions of each plant type were normalized to 100% individually, error bars denote standard errors.](image)

![Figure 10. Green leaf volatiles emitted by wounded phloem from mini seedlings.](image)

The enantiomeric separation of α-pinene and limonene from the headspace of wounded conventional seedlings did not show any differences in enantiomeric composition to the composition before wounding (t-test: α-pinene p = 0.10, limonene p = 0.53). The proportions of the enantiomers in the emission from the needles of unwounded plants seemed to be similar to the proportions of the compounds in the phloem of the plant. After wounding of the conventional seedlings, the emission of both limonene and α-pinene increased, however, to different extent. The attractive α-pinenes increased from being a small part of the volatile blend to being the most dominant compounds (38%).

The previously observed increased in attraction by the large pine weevil to wounded plants (Tilles et al., 1986, Nordlander, 1991) can be explained by the large increase of terpenes released from the conventional seedlings observed in this study. Especially the large increase of both enantiomers of α-pinene in comparison with the smaller increase of the enantiomers of limonene can be of importance. Mini seedlings are sensitive to wounding, but once wounded they may not become more attractive to the large pine weevil. Instead of the attractive monoterpenes that are released by the conventional plants, the mini seedlings mainly emitted green leaf volatiles. The green leaf volatiles are generally regarded to be non-host compounds to conifer associated bark beetles, including the pine weevil since the GLVs are more characteristic for
grass and deciduous trees than for conifers, and can be used as repellents to several bark beetle species (Zhang and Schlyter, 2004).

The differences in emissions of unwounded plants might explain the larger attractiveness of the older conventional seedlings to the large pine weevil. The repellent (S)-(−)-limonene were the most dominant compound in the volatiles of both mini and conventional unwounded seedlings, but represented a larger proportion of the mini seedling’s volatiles. The older plants seemed to emit a larger total amount of volatiles and in addition the volatiles contained the attractive enantiomers of α-pinene.

Evidently the mini seedlings will grow and develop the chemical pattern of the conventional seedlings and then their attraction to the large pine weevil should increase. However, larger plants are less sensitive to damage of the large pine weevil (Thorsén et al., 2001). In addition, naturally regenerated seedlings can endure pine weevil feeding better than planted ones (Selander et al., 1990). The mini seedlings resemble the naturally regenerated plants since they have a rapid root establishment (I), which should increase their vigour and resistance, as compared with conventional seedlings. Variations may exist at what age seedlings will start to produce terpenes. Seedlings with a delayed terpene production can be of interest in conifer tree breeding programs.
The volatile profile of spruce plants differ between ages and individual plants. In addition, the emission changes when a plant is under stress. The factors eliciting the stress reactions in plants can be both abiotic (draught, water, ozone, wounding) and biotic (insect feeding, egg deposition, fungal growth). Some of these stress elicitors have been shown to effect the volatile emission of conifers (Priemé et al., 2000, Mumm et al., 2003, Miller et al., 2005, Ormeno et al., 2007) others do not (Lindskog and Potter, 1995).

When the stress response is elicited, a chain of reactions takes place in the conifer which finally leads to an altered biosynthesis of e.g. terpenes emitted to the surroundings (Dudareva et al., 2006, Keeling and Bohlmann, 2006, Phillips et al., 2006). Part of this signalling pathway involves methyl jasmonate (MeJA) which induce stress reaction when applied exogenously on plants and has been used as a synthetic stress elicitor in several studies on conifers (Phillips et al. 2006 and references therein). Synthetic stress elicitors make it possible to study the induced stress reaction and to see if there are correlations between susceptibility to different pests and the dynamics of the induced defence reaction. Knowledge about the chemical defence and susceptibility of individual plants is of interest in conifer tree breeding programs.

We have studied the volatile response of Norway spruce plants to synthetic, abiotic and biotic stress elicitors.

Nature of the volatile response to different stress elicitors

Response to MeJA

The volatile response to MeJA has previously been investigated in spruce (Martin et al., 2003, Miller et al., 2005). Martin et al. (2003) examined the MeJA induced emissions in plants of two Norway spruce clones, and both similarities and differences were found. Both clones investigated induced the production and emission of linalool, (E)-β-farnesene and (E)-α-bisabolene, whereof (E)-β-farnesene was the most dominant compound. In one of the clones (E,E)-α-farnesene were among the major sesquiterpenes, and could be found in smaller amounts in the other (Martin et al., 2003). In the same study, not only terpenes but also the aromatic phenol methyl salicylate was found in the headspace of MeJA treated plants (Figure 11).

The volatile responses of three Norway spruce clones of known susceptibility to the large pine weevil were followed after MeJA treatment (II). When all emitted volatiles were considered constitutive volatile differences between the clones could be found (Table 1 in II). The differences were too large to separate non-treated and treated plants with multivariate analysis unless the analyses were restricted to the stress-induced compounds.
Figure 11. Compounds emitted by Norway spruce plants after treatment with methyl jasmonate.

The plants showed similar response to the MeJA treatment with increased emission of (E)-β-farnesene, (E,E)-α-farnesene, (E)-α-bisabolene and linalool, with the sesquiterpenes reaching their maximum 12 hours after treatment and linalool more than 24 hours after MeJA application (Figure 12). The induced volatiles and the time course of the reaction were thus similar for the three clones but the magnitude of the response and the constitutive volatiles differed between them. Methyl salicylate was only detected in two of the clones, 72 and 1090, but was present already before MeJA application and did not show a clear pattern of induction.

Figure 12. Emission of (E)-β-farnesene and linalool after MeJA treatment from plants of three different clones: clone 72 (△), clone 1090 (□) and clone 1091 (◇). The time point of peak emission differs between the two compounds.
Since MeJA induces change in volatiles as well as resin compositions in conifers, an effect on the interactions with insects is expected. Application of MeJA on Scots pine seedlings increase the resistance against the large pine weevil (Heijari et al., 2005) but the same effect has so far not been found in Norway spruce plants (Bo Långström personal communication). The same is true for the interactions with the white pine weevil, *Pissodes strobi* (Nicole et al., 2006). However, Norway spruce gains resistance against the bark beetle *Ips typographis* after MeJA treatment (Erbilgin et al., 2006), and MeJA induction also increases the resistance against pathogenic fungi (Kozlowski et al., 1999, Franceschi et al., 2002, Zeneli et al., 2006). But the increased resistance is not for free, the plants pay the cost with reduced growth and photosynthesis (Heijari et al., 2005).

Response to aphids and other herbivore organisms (III)

Previous research of volatile response in conifers to herbivory have mainly focused on monoterpene emission (Litvak and Monson, 1998, Priemé et al., 2000) but recently the induced emission of sesquiterpenes and linalool has gained interest. Miller et al. (2004) discovered that, in addition to passively released resin volatiles, white pine weevils induce a diurnal emission of mainly \((R)-(\text{-})\)-linalool in Sitka spruce (*Picea sitchensis*). Other compounds induced were \((Z)\)-\(\alpha\)-bisabolene and \((E,E)\)-\(\alpha\)-farnesene and the induced emissions were similar to the ones induced by MeJA in the same study. Oviposition by the sawfly *Diprion pini* induced the release of \((E)\)-\(\beta\)-farnesene by Scots pine (Mumm et al., 2003) and attracted a parasitiod of the sawfly (Hilker et al., 2002, Mumm and Hilker, 2005).

The spruce shoot aphid *Cinara pilicornis* is seldom considered as a pest and its performance on Norway spruce has mainly received interest as a biological predictor of air pollution (Holopainen et al., 1993, Kainulainen et al., 1993, Holopainen et al., 1995, Holopainen and Kossi, 1998, Viskari et al., 2000a, Viskari et al., 2000b). We studied their released chemicals and their induction of volatiles from *P. abies* plants.

Norway spruce plants infested with the spruce shoot aphid *Cinara pilicornis* emitted similar compounds as MeJA-treated plants did (Figure 13). In addition to the stress-induced volatiles, *cis-trans*-nepetalactone was present in the odour blend. This compound together with *cis-trans*-nepetalactol and citronellol was not emitted by the plants but were shown to come from the aphids (Figure 13; II). *cis-trans*-Nepetalactone and *cis-trans*-nepetalactol function as sex pheromones in several aphid species (Dawson et al., 1990, Birkett and Pickett, 2003). Nevertheless, it has not been described in species of the genus *Cinara* before. Although no behavioural studies have been performed on the spruce shoot aphid and the effect of *cis-trans*-nepetalactone or the alcohol, it is probable that the compounds have a pheromone function in this aphid species. The discovery that the compound was only emitted during the autumn period (October-November) and not during other seasons supports this hypothesis (III).

Since several aphid species use the same compounds as pheromones, proportions of the pheromone components and host plant volatiles could be important for aphids to find their conspecifics (Powell and Hardie, 2001). The three major induced compounds in aphid infested spruce plants were \((E)\)-\(\beta\)-farnesene, \((E,E)\)-\(\alpha\)-farnesene and methyl salicylate. Methyl salicylate (MeSA) was the compound most induced by artificial spruce shoot aphid infestation on Norway spruce seedlings (III). MeSA has recently been shown to increase the response of male aphids of the species *Rhopalosiphum padi* and *Phorodon humuli* to their respective nepetalactol sex pheromones (Pope et al., 2007). The compound has long been associated with stress...
signalling pathways in plants, (see e.g. Arimura et al., 2005). It had an antiaggregant effect for *P. humuli* during spring (Campbell et al., 1993) and at high doses MeSA acts as antifeedant for the large pine weevil (Borg-Karlson, personal communication).

**Figure 13.** Chromatograms of headspace samples of aphids (top), Cinara-infested (middle) and uninfested (bottom) Norway spruce plants of clone 1321. Three compounds were found in the headspace of *Cinara plicicornis*, the other peaks were due to the background. In the chromatogram of plants with aphids three stress-induced compounds are marked out, these were not present in the volatiles of the uninfested plant.

Many aphid species use *(E)-β*-farnesene as an alarm pheromone (Pickett and Griffiths, 1980, Xiangyu et al., 2002) but in behavioural tests with two other *Cinara* species no alarm behaviour could be detected (Xiangyu et al., 2002). The spruce shoot aphids excreted alarm associated cucurbit drops at some occasions during headspace sampling, but no *(E)-β*-farnesene was found (III). It seems likely that the conifer feeding *Cinara* genus do not use *(E)-β*-farnesene as alarm pheromone and it is interesting to speculate whether this might be the cause of coevolution between conifers and their associated aphids. *(E)-β*-Farnesene are induced by a number of plants upon stress (Paré and Tumlinson, 1999) and wild potato (*Solatium berthaultii* Hawkes) gained increased resistance to aphids by release of the compound (Gibson and Pickett, 1983). But there are also examples of aphids which can distinguish between the plant derived *(E)-β*-farnesene and the alarm pheromone emitted by conspecific aphids by recognizing other plant volatiles. *(−)-β*-Caryophyllene or volatiles from uninfested hop plants released together with *(E)-β*-farnesene inhibited the effect of the compound as an alarm pheromone for three aphid species (Dawson et al., 1984).
Herbivores with different feeding behaviour can elicit separate reactions in plants. On the gene level Ralph et al. (2006) found both similarities and differences in the up- and down regulation of genes in Sitka spruce (*Picea sitchensis*) due to feeding by western spruce budworm (*Choristoneura occidentalis*) and the white pine weevils (*Pissodes strobi*). It could be of advantage for the tree if the volatile responses differ depending on herbivore since it could facilitate the attraction of predatory insects or parasites specific for the herbivore.

**Figure 14.** PCA biplot based on headspace samples of Norway spruce plants of clone 1321 infested with different organisms: *Nalepella*-infested plants, ◇; spider mite-infested plants, □; *Cinara*-infested plants, ×; and plants after feeding of *Hylobius abietis*, △. Arrows indicate directions of increasing relative amounts of the stress-induced compounds.

In a minor study (unpublished) we analysed the volatiles of 2-3 years old spruce plants of clone 1321, separately infested with four different herbivore species: *Nalepella haarlovi* Boczek var. piceae-abietis Löytyniemi, spruce spider mites, spruce shoot aphids (*Cinara pilicornis*) and large pine weevils (*Hylobius abietis*). Multivariate analysis was performed on the relative compositions of selected stress-induced compounds collected from the headspace of the plants. PCA separated the samples into groups according to herbivore (Figure 14), indicating that although the four herbivore species elicited similar compounds to be emitted; the proportions of the compounds differed.

The variable vectors indicate a possible correlation between compounds. The two α-farnesenes do not show any signs of correlation with each other, instead (3Z, 6E)-α-farnesene seems to correlate with (E)-α-bisabolene. (E)-α-bisabolene synthases have been identified in Norway spruce but only producing one single product (Martin *et al.*, 2004). However, (E)-α-bisabolene has also been found as a product from the non-specific γ-humulene synthase in *Abies grandis* (Steele *et al.*, 1998) so it is possible that the two compounds are indeed produced by the same enzyme. The same can not be true for (E,E)-α-farnesene and methyl salicylate since the latter is produced by the shikimic acid pathway. If a true correlation exists this is due to simultaneously activation of enzymes of different biosynthetic pathways in the plant.
The feeding behaviour of the pine weevils differ from the other herbivores. Both mites and aphids species suck their nourishment from the plant, while the pine weevil feed on the bark of the plant creating wounding of the stem. The wounding liberates volatiles from the constitutive resin into the air giving the plant a mixed odour of resin terpenes and terpenes emitted by the needles.

Response to mechanical wounding

The volatiles of Norway spruce plants after wounding are dominated by emission of terpenes from the resin (I; II; Miller et al., 2005). However, conifers also respond to mechanical wounding in a similar way as to MeJA treatment or insect feeding on the gene expression level (Ralph et al., 2006). Repeated mechanical wounding of the stem of a Norway spruce plant induced the emission of stress compounds such as (E)-β-farnesene, (E,E)-α-farnesene, methyl salicylate and (R)-(−)-linalool (Kännaste, personal communication).

To distinguish the stress-induced terpenes from the ones emitted from the wound is difficult. One way is to take a detour over the enzymes and study which transcriptions are induced (Martin et al., 2003, Miller et al., 2005). From Martin et al. (2003) we know that in Norway spruce the main products of activated TPS in the needles are emitted to the surroundings, however, there are also terpene pools within the needles which increase their content after stress induction. It has been suggested that the biosynthesis of stored and emitted terpenes may occur at separate locations in the needle (Martin et al., 2003) but this remains to be shown. Until then it is not possible from TPS activity alone to know which needle produced compounds are released to the surroundings and which are stored in the needle. Another way to distinguish the induced volatile terpenes from the constitutive ones is by careful analysis of volatiles from different sites of the plant (II). A miniaturized sampling method is then needed to further perform detailed studies on the emission and effects of behaviour mediated volatiles on arthropods.

Localization of the induced volatile response (II)

A stress elicitor gives rise to a reaction at the site of attack, but can also induce a systemic response. The systemic responses prepare other parts of the plant for a possible spreading of the cause of stress (e.g. feeding insects, fungal pathogen). So far one volatile example has been found among conifers: the systemic volatile indirect defence of Pinus sylvestris against the pine saw fly (Diprion pini) (Hilker et al., 2002, Mumm et al., 2003).

Two-year old Norway spruce seedlings (conventional ones, for planting in reforestation) were applied with MeJA (1:100) on the five lowest centimeters of the stem by a soft brush. Two needles on the treated areas together with two needles growing above (one and two centimetres up, respectively) were analysed with the single needle setup. Needles growing on MeJA-treated areas emitted the induced mixture of sesquiterpenes and linalool previously described. These compounds were absent in the headspace of the needles growing above the MeJA-treated area, only one of the needles emitted small amounts (Figure 15). The clear difference indicated a highly localized response immediately after MeJA treatment. This could explain the quantitative difference in response between the clone plants investigated (Figure 12), it was possible that the magnitude of the response was related to the number of needles growing on the MeJA-treated part of the stem.
A short distance systemic response was previously found in the anatomical response of *Picea abies* to MeJA application (Franceschi *et al.*, 2002). Franceschi *et al.* used a similar application technique and MeJA induced the formation of traumatic resin ducts (TDs) and polyphenolic parenchyma cells (PP cells). The PP cells and TDs formed above the treated area had features indicating a later and weaker induction of formation than at the MeJA-treated site. In previous investigations of volatile emission from conifers after MeJA-application, the whole plants were sprayed with MeJA solution, thus not making it possible to investigate systemic responses (Martin *et al.*, 2003, Miller *et al.*, 2005).

Volatile MeJA or induced ethylene from MeJA-treated stem was not of sufficient concentration to penetrate the cambium and induce anatomic defences; these were considered to be due to signal transduction within the phloem and xylem (Franceschi *et al.* 2002, Hudgins and Franceschi 2004). However, in our case the volatiles analysed were produced and emitted by the more exposed needles and it was possible that volatiles from the applied MeJA could enter the needles and influence the terpene production located there. None of the needles on the untreated area had MeJA in their headspaces. Of the needles growing on the treated areas, no correlation between the amounts of MeJA and the amounts of emitted (E)-β-farnesene could be found (Figure 16).
MeJA induce the production of ethylene, which is regarded to be the signal compound responsible for the local and systemically induced defences in conifers (Hudgins et al., 2004, Hudgins and Franceschi, 2004, Ralph et al., 2006). Ethylene was not detected with the single needle methodology but cannot be ruled out. It is therefore possible that the signal inducing the needle response reaches the needle either as a volatile from the outside, or through the signal network within the plant.

Wounding also gives rise to volatile responses but as mentioned there are difficulties in separating the induced response from the passive emission of terpenes from the wound. The single needle setup was used to shed light on the response to wounding. The emissions of a needle growing above a wounding site were compared with the emissions from a needle growing on the opposite stem side as well as the emission from the released resin (II).

The volatile blend from the resin consisted of high amounts of monoterpenes and these also diffused into the glass tubes over the needles. However, since the main stress-induced volatiles belong to the larger sesquiterpenes the analyses were focused on this compound group in both resin and needle headspace samples. (E)-β-Farnesene, (E,E)-α-farnesene and (E)-α-bisabolene could be found in the headspace of the needle at the wounding site after wounding (needle 1), but for the other needle (needle 2), as well as for both needles before wounding, they were only found in trace amounts in a few cases (Figure 17). To elucidate if the compounds in the headspace of needle 1 were indeed stress-induced emissions or if they were part of the compounds released from the wound diffusing into the glass tube, a more thorough investigation of the sesquiterpene composition in the different samples was performed.

When measuring the headspace of resin and a needle near to the wounding site, there were huge differences in the number of sesquiterpenes detected. The headspace of the resin samples contained 12-29 sesquiterpenes, the needles at the wounding site 4-28 sesquiterpenes while the needles on the opposite side of the stem did not emit sesquiterpenes of detectable amounts, except for one with 7 sesquiterpenes in its headspace. When comparing the relative amounts of (E)-β-farnesene, (E,E)-α-farnesene and (E)-α-bisabolene, distinct differences between the resin and the needle at the wounding site could be seen (Figure 17). (E)-β-Farnesene made out a smaller proportion of the resin volatiles than of the needle volatiles, the needle volatiles also contained substantial amounts of (E,E)-α-farnesene and some (E)-α-bisabolene. If the presence of farnesenes and bisabolene in the needle headspace was due to diffusion of resin emissions, no differences in composition should have been found between the needle and the resin headspace. These dissimilarities suggested that the compounds were produced and released by the needle as a response to the mechanical wound in the nearby stem.
The single needle setup makes it possible to localize the site of stress by the induced terpene emission of needles. With this methodology it could be possible to follow the volatile response to needle living mites or the growth of a fungal pathogen within the plant (if volatiles are induced by its advancement in the plant).

Figure 17 Relative amounts of stress-induced compounds in the headspace of resin, needle at wounding site (needle 1) and needle at opposite side of stem (needle 2). The sum of all sesquiterpene peak areas was normalized to 100% for each chromatogram individually, only the three sesquiterpenes associated to stress are showed. Five plants were analyzed; error bars denote standard errors.
Conclusions

- There exist chemical differences between plants of different ages, both in the volatiles emitted from intact seedlings and the volatiles released by wounded phloem.

- Among the volatiles produced by Norway spruce plants of different ages (S)-(−)-limonene and the green leaf compounds were possible candidates of resistance markers for mini seedlings against *Hylobius abietis*.

- It was possible to study the emission of single needles *in vivo* during stress response with SPME and the “single needle setup”. A local response to the stress elicitor methyl jasmonate and to mechanical wounding was detected by this technique.

- The induced volatiles of the Norway spruce plants to different stress elicitors mainly consisted of five compounds: (R)-(−)-linalool, methyl salicylate, (E)-β-farnesene, (E,E)-α-farnesene and (E)-α-bisabolene. Among these, methyl salicylate showed a clear antifeedant effect when tested on feeding *Hylobius abietis*.

- Three clones with different susceptibility to *Hylobius abietis* had different volatile profiles but had similar dynamics in the induced volatile response to MeJA treatment.

- SPME was also suitable to collect volatiles from live spruce shoot aphids and during short autumn periods nepetalactone, nepetalactol and citronellol were found in the headspace of the aphids.

- A method to separate and identify the four diastereomers of nepetalactone by GC-MS has been developed.
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If you read the thesis you know that farnesenes play a central role in the induced volatile stress response of Norway spruce and I would like to thank Dr. Ilme Liblkas for providing α-farnesene references.

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Appendix

Description of the author’s contribution to manuscript I-III

I. Performed the GC-MS analyses and wrote the major part of the chemistry sections in the manuscript.

II. Performed all experimental work and wrote the manuscript

III. Performed the experimental work except the nepetalactone analyses on MS-instruments other than the Finnigan SSQ 7000. Made all calculations and wrote the manuscript.