Chemical Defence in Norway Spruce

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Doctoral Thesis
Stockholm 2011
Abstract

Norway spruce (*Picea abies*) responds to stress by biosynthesis of chemical substances, which can deter invading insects or pathogens. Some of these substances are volatile and can be emitted to the surroundings while others are accumulated within the tree. 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.

The first part of this research focused on volatiles emitted by Norway spruce plants. Collection of headspace volatiles by SPME and subsequent separation and identification with GC-MS was 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 spatial localisation of the stress reaction to methyl jasmonate or spruce spinning mites. Seedlings of different ages showed differences in chemical composition of emitted volatiles, with the pine weevil repellent, (4S)-(-)-limonene, 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. In addition, a method to separate and identify the four diastereomers of nepetalactone by GC-MS and characteristic m/z-fragments was accomplished.

The second part of the research deals with the chemical response of Norway spruce roots to inoculation with *Heterobasidion annosum*. Terpene concentrations increased after inoculation or wounding but the composition was mainly associated with clone identity and not to susceptibility or treatment. In contrast, inoculation with *H. annosum* induced a treatment-specific alteration of phenol composition. The constitutive phenol composition differed between more and less susceptible clones. The phenols astringin and astringin dimers (piceasides) as well as the terpene α-longipinene may be suitable markers of low susceptibility for *P. abies* to *Heterobasidion*.

Keywords: *Picea abies*, *Hylobius abietis*, *Cinara pilicornis*, *Oligonychus ununguis*, *Heterobasidion annosum*, volatiles, terpenes, green leaf volatiles, stilbenes, stress response, nepetalactone.
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This thesis is based on the following papers and manuscripts, which will be referred to by their Roman numerals I-IV.

I. **Semiochemicals related to the aphid *Cinara pilicornis* and its host, *Picea abies*: A method to assign nepetalactone diastereomers.**
   *Journal of Chromatography A*, 2008, 1180: 165-170

II. **Mini-seedlings of *Picea abies* are less attacked by *Hylobius abietis* than conventional ones: Is plant chemistry the explanation?**
   Marie Danielsson, Astrid Kännaste, Anders Lindström, Claes Hellqvist, Eva Stattin, Bo Långström and Anna-Karin Borg-Karlson.

III. **Tracing induced stress sites in conifers by single needle analyses.**
    Marie Danielsson and Anna-Karin Borg-Karlson.
    *Manuscript*

IV. **Chemical and transcriptional responses of Norway spruce clones with different susceptibility to *Heterobasidion* spp. infection.**
   Marie Danielsson, Karl Lundén, Jenny Arnerup, Jiang Hu, Tao Zhao, Gunilla Swedjemark, Malin Elfstrand, Anna-Karin Borg-Karlson and Jan Stenlid.
   *Preliminary manuscript*

During the course of this work, I have changed my family name from Pettersson (paper I) to Danielsson.

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**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>2D</td>
<td>Two dimensional</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>d.p.i.</td>
<td>Days post inoculation</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DVB</td>
<td>Divinylbenzene</td>
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<tr>
<td>DW</td>
<td>Dry weight</td>
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<tr>
<td>EI</td>
<td>Electron ionisation</td>
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<td>er</td>
<td>Enantiomeric ratio</td>
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<tr>
<td>ESI</td>
<td>Electrospray ionisation</td>
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<tr>
<td>GC</td>
<td>Gas chromatography</td>
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<td>GLV</td>
<td>Green leaf volatile</td>
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<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
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<tr>
<td>LAR</td>
<td>Leucoanthocyanidin reductase</td>
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<tr>
<td>LC</td>
<td>Liquid chromatography</td>
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<tr>
<td>LOX</td>
<td>Lipoxygenase</td>
</tr>
<tr>
<td>MeJA</td>
<td>Methyl jasmonate</td>
</tr>
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<td>MEP</td>
<td>Methyl-D-erythritol phosphate</td>
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<tr>
<td>MS</td>
<td>Mass spectrometry</td>
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<tr>
<td>MVDA</td>
<td>Multivariate data analysis</td>
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<tr>
<td>m/z</td>
<td>Mass to charge ratio</td>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>PaTPS-Car</td>
<td>(+)-3-Carene synthase gene (from <em>Picea abies</em>)</td>
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<tr>
<td>PaTPS-Lim</td>
<td>(-)-Limonene synthase gene (from <em>Picea abies</em>)</td>
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<tr>
<td>PC</td>
<td>Principal component</td>
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<td>PCA</td>
<td>Principal component analysis</td>
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<td>PDA</td>
<td>Photo diode array</td>
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<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
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<tr>
<td>PP-cell</td>
<td>Polyphenolic parenchyma cell</td>
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<tr>
<td>RDA</td>
<td>Redundancy analysis</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>SEK</td>
<td>Swedish krona</td>
</tr>
<tr>
<td>SPME</td>
<td>Solid phase microextraction</td>
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<tr>
<td>TPS</td>
<td>Terpene synthase</td>
</tr>
<tr>
<td>s.l</td>
<td><em>Sensu latu</em></td>
</tr>
<tr>
<td>s.s.</td>
<td><em>Sensu stricto</em></td>
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<tr>
<td>spp.</td>
<td>Species (plural)</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>VOC</td>
<td>Volatile organic compound</td>
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1. Introduction

1.1 Aim
The aim of my research is to investigate the chemical response of Norway spruce (*Picea abies* (L.) Karst.) to different types of biological stress factors. The focus is on the production and emission of low molecular weight metabolites, with potential semiochemical function, using trees of various ages and genotypes.

1.2 Background
Conifers are long-lived trees belonging to the gymnosperms, which include 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 importance. In Sweden 80% of the forests consist of conifers, mainly Norway spruce and Scots pine (*Pinus sylvestris*) (Fransson 2010).

The life of a conifer is nevertheless filled with challenges. There are a number of insects and other arthropods associated with conifers; those that cause economical damage are defined as pests (Berryman 1982). The organisms themselves may be the main cause of damage to the plant, or they can also be vectors for fungal pathogens and other diseases. One of the reasons for conifers successful survival is the production of a variety of terpenes and other metabolites, which differ in composition between trees of the same species (Lieutier et al. 2003; Persson et al. 1996; Slimestad 1998). The variations in chemical composition may lead to differences in resistance or susceptibility to insects and pathogens, and the substances responsible for these differences can be constitutive compounds as well as products of induced defence reactions (Almquist et al. 2006; Brignolas et al. 1998; Franceschi et al. 2005).

1.2.1 Conifer chemical defence strategies
Terpenes and phenols are continuously produced by the plants of the family Pinaceae. The compounds are stored in special structures, such as resin ducts and polyphenolic parenchyma cells (PP-cells), within the tree (Franceschi et al. 2005). The constitutive terpenes function as a defence against invading insects. During mechanical wounding the resin ducts get punctured and resin flows out, deterring the insect and sealing the wound (Phillips and Croteau 1999).

In addition, conifers possess an induced chemical defence (Figure 1); biotic, abiotic and synthetic stress elicitors activate biosynthetic pathways in
the tree (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 PP-cells (Franceschi et al. 2002; Franceschi et al. 2005) as well as altered quantities and compositions of both terpenes (Fäldt et al. 2006; Phillips et al. 2006; Sjödin et al. 1993) and phenols (Evensen et al. 2000; Franceschi et al. 2005; Viiri et al. 2001). If the induced defence is effective, the newly produced compounds are more toxic to the invader than the constitutive ones and stop its feeding or colonisation of the tree. The initial induced response is commonly localised at the site of attack but it can also give rise to a systemic response within the plant, preparing other parts to respond to potential attacks by the aggressor.

The stress-induced changes do not only occur within the trees. Conifers release volatiles, mainly terpenes, from their needles and the quantitative and qualitative composition of the emitted terpenes change when trees are stressed (Martin et al. 2003; Miller et al. 2005). This odour change 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).

Figure 1. Overview of the chemical defence in a tree. Aggressors elicit the activation of defence related biosynthetic pathways of the tree, which lead to the production of metabolites that can affect the aggressor in a direct or indirect way.

This thesis focuses on the low molecular weight metabolites of Norway spruce that may interact with organisms challenging the health of the tree. To provide information on how these compounds are produced, the next section will give a brief description of their biosynthesis.
1.3 Biosynthesis of conifer metabolites

The allocation of carbon resources in a plant is often described from the growth or defence theory (Gershenzon 1994; Herms and Mattsson 1992), i.e. either the plant uses its resources to grow or to produce defence molecules. Figure 2 shows a simplified scheme of the relationship between the biosynthetic pathways leading to the compound classes studied in this thesis. They all originate from products of glycolysis, where glucose breaks down to acetyl-CoA, which either is used for metabolism of new compounds or enters the energy producing citric acid cycle of the mitochondria. The biosynthetic pathways leading to phenols, terpenes and green leaf volatiles are briefly described below.

![Figure 2. Overview of the biosynthetic pathways that lead to the compound classes studied in this thesis (marked in bold). Abbreviations: PEP: phosphoenolpyruvate, MEP: methyl-D-erythritol phosphate, MVA: melvanoate, LOX: lipoxygenase.](image)

1.3.1 Phenol biosynthesis

Many phenolic compounds in plants, such as stilbenes and flavonoids as well as the structural polymer lignin, are synthesised from phenylalanine through the phenylpropanoid metabolism (Dixon and Paiva 1995; Dudareva et al. 2004). The phenylpropanoid metabolism is a complicated network of reactions and enzyme activities with its starting point at the end of the shikimic acid pathway (Dixon et al. 2001). Figure 3 shows the first common steps and some of the product classes obtained from the biosynthetic pathways.
The stilbenes and flavonoids have been the target of much research on interactions between conifers and their pests, particular bark beetles and associated fungal pathogens (Brignolas et al. 1998; Franceschi et al. 2005; Lieutier 2002; Ralph et al. 2006).

Stilbenes are formed by stilbene synthases (STSs) from \( p \)-coumaroyl-CoA or structural analogues. They can then go through further modification such as isomerisation, methoxylation, glycosylation and oligomerisation as denoted in Figure 4 (Chong et al. 2009). The glycosylation is an important step since it makes the stilbenes water soluble and more easily stored and transported within the plant tissue.

**Figure 3.** The first central steps of phenylpropanoid metabolism in plants. Enzymes abbreviations: PAL: phenylalanine ammonia-lyase, C4H: cinnamate 4-hydroxylase, 4CL: 4-coumarate CoA-ligase, STS: stilbene synthase and CHS: chalcone synthase.

**Figure 4.** The formation of stilbenes through stilbene synthases (STS) and some examples of common transformations the compounds may undergo. The stilbenes mentioned are those commonly found in conifers and their drawn structures can be found in Appendix I. Modified from Chong et al. (2009).
1.3.2 Terpene biosynthesis

Terpenes are produced through two different biosynthetic pathways. Sesquiterpenes are formed through the melvanoate (MVA) pathway in the cytosol; monoterpenes and diterpenes via the methyl-D-erythritol phosphate (MEP) pathway in the plastids as shown in Figure 5 (Rohmer 1999). Both pathways give rise to the substrates of terpenes: isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) and exchanges of these compounds occur between the cytosol and the plastid (Dudareva et al. 2004; Rohmer 1999). They condense and form the precursors of monoterpenes (geranyl diphosphate), sesquiterpenes (farnesyl diphosphate) and diterpenes (geranylgeranyl diphosphate) which are then transformed by terpene synthases (TPSs) to the various terpene products of the plant. The diterpenes are thereafter further functionalised into resin acids by cytochrome P450-dependent monoxygenases located in the endoplasmic reticulum (Zulak and Bohlmann 2010).

In the last decades a lot of work has been devoted to identify and characterise the TPSs of conifers and so far, ten have been functionally characterised for Norway spruce (for reviews see: Keeling and Bohlmann 2006; Zulak and Bohlmann 2010). Some TPSs give rise to highly specific products (e.g. Bohlmann et al. 1998) while others produce a multitude of products (e.g. Steele et al. 1998). A number of TPS transcripts are up-regulated upon stress, leading to induced biosynthesis of terpenes (Huber et al. 2004; Keeling and Bohlmann 2006; Phillips et al. 2007).

**Figure 5.** Biosynthesis of terpenes occurs through two different biosynthetic pathways. MEP: methyl-D-erythritol phosphate, MVA: melvanoate, DMAPP: dimethylallyl diphosphate, IPP: isopentenyl diphosphate, GPS: geranyl diphosphate synthase, GGPS: geranylgeranyl diphosphate synthase, FPS: farnesyl diphosphate synthase and TPS: terpene synthase. Modified from Tholl (2006).
1.3.3 Green leaf volatile biosynthesis

Metabolites of the lipoxygenase (LOX) pathway are 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 6) (Blee 1998). Green leaf volatiles (GLVs) are comprised 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 membrane lipids are mixed with enzymes liberating the fatty acids, which supply the substrates for GLV biosynthesis (Matsui 2006; Paré and Tumlinson 1999). 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 6](image.png)

**Figure 6.** Part of the lipoxygenase pathway. Jasmonates, green leaf volatiles and traumatic acid are all compounds associated to stress in plants. LOX: lipoxygenase, AOS: allene oxide synthase, JMT: jasmonic acid methyl transferase, HPL: hydroperoxide lyase and ADH: alcohol dehydrogenase. Adapted from Hatanaka (1993) and Matsui (2006).

The green leaf volatiles, terpenes and phenols can all play a role in the defence mechanisms of conifers. In the following chapters the production, emissions and accumulation of these compounds by Norway spruce under different conditions are investigated.
1.4 This study

This thesis explores the chemical defence of Norway spruce and its interactions with fungi and insects. Responses to various stressors are studied, new methods are developed and correlations to observed natural variation in susceptibility to different aggressors are sought.

The next chapter gives a short overview of the methods available to ecological chemists today and the methods used in this thesis. I present a new way to assign nepetalactone diastereomers by their mass spectra and gas chromatography retention indices (I). The identification of piceasides, which are further discussed in chapter 5, are also accounted for.

Observations show that the large pine weevil prefers seedlings between the ages of 1-2 years to the so called mini seedlings only 6-10 weeks old. In chapter 3, the volatile profiles of seedlings of these ages are compared, in both unwounded condition and emissions released by wounding (II).

Chapter 4 continues to deal with the volatile emissions of Norway spruce plants, seedlings and cuttings 1-4 years old, and how their emission changes in response to stress. The chemical response to spruce shoot aphid infestation is investigated and the aphid pheromone is reported in section 4.1 (I). In section 4.2 the spatial localisation of the volatile responses to methyl jasmonate and spinning mite infestation are investigated by head space-SPME of individual needles (III). The magnitude of the response and possible contribution to atmospheric VOC’s are discussed in section 4.3.

In the 5th chapter, chemical responses to fungal inoculation and wounding within roots of Norway spruce trees are studied. Relations between constitutive chemical profiles and observed variations in susceptibility to *Heterobasidion* spp. between clones are investigated (IV).
The field of biochemistry has developed tremendously during the past decades and new tools and knowledge have given us increased possibilities to understand the processes in plants, both in different stages of development and in their reactions to stress. Early work was mainly performed on herbaceous plants such as *Arabidopsis*, maize and tobacco. Among trees, poplar was the first model species and its genome has been sequenced (Tuskan et al. 2006).

The study of conifers is now taking speed. Several enzymes encoding terpene and phenol production have been identified and a protein data base for the spruce genus is under development (Lippert et al. 2009). The genome of Norway spruce is currently being sequenced by researchers at the Umeå Plant Science Center (www1 2010) which will further increase our knowledge of this species.

Biochemical techniques have been used to study conifer response to stress. Ralph et al. (2006) made a large scale cDNA microarray on Sitka spruce and applied it to the study of mechanical damage and feeding by two insect species. Several thousand defence genes were revealed and some differences in expression levels between inducers were found.

However, for complete understanding the metabolic products of the trees’ biochemical activity also need to be analysed. Martin et al. (2003) showed that in Norway spruce the main products of activated TPSs in the needles were emitted to the surroundings. Nevertheless, there were also terpene pools within the needles, which increased their content after stress induction. It was 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.

Presently 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. In order to decipher the signals a plant sends out, it is necessary to collect and analyse the compounds from its headspace, i.e. the air above and around the plant. Another factor that can lead to discrepancies between the expressed genes and chemical content is the substrates available to the enzyme. Stilbene synthases can accept different substrates, e.g. pinosylvin synthase use both cinnamoyl-CoA and caffeoyl-CoA *in vitro* to produce pinosylvin and piceatannol, respectively (Raiber et al. 1995).

Just as in biochemistry, there has been an intense development within the field of analytical chemistry. In a few decades the commercialisation of two dimensional gas chromatography (2DGC) coupled to mass spectrometry (MS),
and liquid chromatography coupled to mass spectrometry (LC-MS) have increased the availability of these methods. In addition, techniques such as matrix-assisted laser desorption ionisation (MALDI), LC-NMR (liquid chromatography-nuclear magnetic resonance) and cryo-NMR have improved the study of plant metabolites (Li et al. 2007; Li et al. 2008b; Wolfender et al. 2003). Now it is even possible to study the metabolites of single cells in vitro (Li et al. 2007; Shrestha and Vertes 2009) and to do in vivo spatial studies of plant contents with laser ablation electrospray ionisation, LAESI (Nemes and Vertes 2007).

2.1 Biological material

The studies were carried out on Norway spruce (Picea abies (L.) Karst.) of various ages. The young seedlings, referred to as mini seedlings, were six to ten weeks old and the conventional seedlings were between one and two years old (II). Clones show less variation in terpene composition than seedlings because of their identical genetic setup (Hanover 1992; Silvestrini et al. 2004). This makes them suitable for use in comparative studies to minimise variation between plants and to find resistance mechanisms coupled to genetic heritage. The clones used in I and III were two to three years old cuttings from archives of Skogforsk (the Forestry Research Institute of Sweden) with known resistance to the large pine weevil (Jan Weslien, personal communication). Clones originating from Skogforsk cuttings were also studied in IV. The trees were 27 years old with known susceptibilities to natural infestation with Heterobasidion spp. (Karlsson and Swedjemark 2006) and were growing at a plantation at Årdala, Sweden (59°01' N, 16°49' E).

Spruce shoot aphids (Cinara pilicornis Hartig) (I) and spruce spinning mites (Oligonychos ununguis Jacobi, Acari: tetranychidae) (III) were obtained from naturally infested plants. The inoculation study (IV) was performed with a strain of Heterobasidion annosum (Sä 16-4) (Stenlid and Karlsson 1991).

2.2 Sample preparation

This work has focused on insect – host tree interactions, where insects can use volatile cues to find their host and evaluate its suitability, as well as interactions with fungi - which can be affected by the chemistry within the tree. Thus, both headspace samples and bark extracts have been prepared and analysed.

2.2.1 Collection of headspace volatiles, solid phase microextraction (SPME)

Several methods to collect headspace volatiles are used in ecological chemistry (Agelopoulos and Pickett 1998; D'Alessandro and Turlings 2006; Millar and
Sims 1998) whereof solid phase microextraction (SPME) is one which has successfully been used in the analysis of headspace volatiles from both plants (I; II; III; Augusto and Valente 2002; Schäfer et al. 1995; Zini et al. 2002) and insects (I; Andersson et al. 2000; Borg-Karlson and Mozuraitis 1996; Moneti et al. 1999).

With SPME, 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 the terpenes emitted by conifers (Fälldt et al. 2000; Mani 1999). 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.

As mentioned previously, the volatiles emitted by plants vary by genetic origin, but temperature, light conditions, season and time of day also affect the emissions (Hakola et al. 2006; Kesselmeier and Staudt 1999; Niinemets et al. 2004; Niinemets et al. 2002; Staudt et al. 2000). 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) of volatiles 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 recognised. Long extraction times contradict the general recommendations set by measuring static headspace samples. However, long extraction times favours the adsorption of larger molecules on the fibre, e.g. (6E)-β-farnesene, one of the key compounds in stress reactions of conifers.

2.2.2 Extraction procedure
To study the chemistry within the tree, bark samples were taken and compounds of interest sequentially extracted from the sample by suitable solvents. Bark plugs were placed directly into vials containing hexane at the sampling site and extracted over night. The hexane extracts were transferred to clean vials and stored in a freezer until analysis of terpene content with GC-MS and 2DGC. The residues were washed again with hexane for one hour and thereafter 80% methanol was added to extract the phenols of the sample, this extraction was also carried out over night and the methanol extracts were transferred to clean vials and kept in a freezer until analysis with LC-MS.
2.3 Sample analysis

In this work, MS was used for detection and identification of compounds and depending on the polarity of the sample, either GC or LC was used to separate the compounds prior to MS. The GC-MS analyses were complemented by separation of monoterpene enantiomers on the 2DGC system, described by Borg-Karlson et al. (1993).

2.3.1 Gas chromatography – mass spectrometry (GC-MS)

Headspace and hexane samples were analysed with a Varian 3400 GC connected to a Finnigan SSQ 7000 MS instrument equipped with electron ionisation (EI, source: 150 °C, 70 eV). The electron ionisation is a hard ionisation technique, which causes the molecules to fragment forming characteristic patterns for each compound. This is useful in compound identification and several commercial spectra libraries exist. In addition to synthetic reference compounds, retention indices and spectra libraries were used for compound identification in this work.

2.3.2 Liquid chromatography – mass spectrometry (LC-MS)

Methanol extracts were separated with a Finnigan HPLC system. To gather as much information as possible about the analytes, a photodiode array detector (Surveyor PDA Plus) was used in series with a MS detector (Finnigan LXQ 2D linear ion trap). The Finnigan LXQ was equipped with electrospray ionisation (ESI).

ESI is a soft ionisation technique and produces mostly molecular ions and various adduct ions. To get more information about molecular structure MS/MS-experiments are needed (i.e. two stage mass analysis). In an MS/MS experiment, a target ion is isolated in the ion trap, excited and fragmented by an applied voltage. The fragmentation patterns are dependent on the voltages used and there are no commercial libraries for low molecular weight plant metabolites; this makes the identification process more difficult compared with the GC-EI-MS technique.

Formic acid (0.1%) was used to facilitate better LC separation and formation of ions. The majority of measurements were performed in negative mode with the ESI source optimised on isorhapontigenin and set up as follows: source voltage 4.00 kV, sheet gas flow 40 au (arbitrary unit), sweep gas 20 au, capillary temperature 270 °C and capillary voltage -23.00 V.

2.4 Statistic analysis of 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 even more can be found within the plant. Moreover, it is not necessarily the most abundant substances that cause the largest ecological impact. To group plants based on their chemical profile all the compounds need to be considered and for this purpose multivariate data analysis (MVDA) is useful (Persson et al. 1996; Silvestrini et al. 2004; Wold et al. 1989).

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 sample scores. The impact of a variable to the PCs increases with its distance to origin. Samples placed close to each other on the score plot have similar variable properties; in this thesis that will typically mean similar chemical composition. 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 the nature of a stress elicitor. The ordination axes of RDA are thus constructed to separate the samples according to the explanatory variables to explain as much of the variation in the data set as possible (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.
2.5 Compound identification

2.5.1 Identification of nepetalactone diastereomers with GC-MS and multivariate analysis (I)

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 data analysis (MVDA) these can be found (Berman et al. 2006; Le Bizec et al. 2005).

We used MVDA to find characteristic fragments of the four diastereomers of nepetalactone (I), of which cis-trans has been characterised as a component of sex pheromones in several aphid species (Birkett and Pickett 2003; Dawson et al. 1990). cis-trans-Nepetalactone was found together with cis-trans-nepetalactol and citronellol in the headspace of spruce shoot aphids 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 a clear impact of concentration on MS-spectra even after the intensities of the m/z-peaks in the spectra had been normalised (maximum peak in each spectra was set to 100%). There was a tendency for the samples to be grouped according to isomer identity, but concentration differences were too large to allow clear grouping. A constrained ordination using 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 PC axis were chosen: 85, 111, 137, 138 and 151. The use of this selection of m/z fragments in PCA gave a plot with four groups corresponding to the four diastereomers (Figure 7).

A simpler test was developed based on these m/z fragments and retention indices (Figure 8) which also worked well with other quadropol instruments (I). To test the method, the natural nepetalactone samples collected from the headspace of the spruce shoot aphid (C. 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 (Figure 7), all aphid samples belonged to this nepetalactone group. This identification could be confirmed by injection on two columns and comparing retention indices with standards.
Figure 7. 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.

Figure 8. Test to assign nepetalactone isomers by retention indices (see Table 1 in I) and comparison of characteristic fragments in MS-spectra. RI: retention index, GC: gas chromatography, MS: mass spectrometry.
2.5.2 Identification of piceasides with HPLC-PDA-ESI-MS

Piceasides are stilbene dimers of isorhapontin and astringin (M\textsubscript{w} = 824 g/mol) or astringin alone (M\textsubscript{w} = 810 g/mol) and eight piceasides have been described in the literature (Li et al. 2008a). According to Li et al. (2008a) the piceasides have different absorbance maxima in UV light, depending on the ring form in the junction between the monomers. The ones with a dihydrofuran ring have the strongest absorbance around 330 nm but the ones with a dihydro-1,4-dioxin ring have strongest absorbance around 323 nm.

We identified the stilbene dimers discussed in IV with ESI-MS, ESI-MS/MS, and UV spectra. Based on full scan MS spectra, ten chromatogram peaks with the m/z fragment 809 or 823 were identified. Comparison of the UV spectra gave one candidate to piceaside A and B, three to piceaside C and D (Figure 9), three to piceaside E and F and three candidates to piceaside G and H (Figure 9). Two of these ten candidates were subjected to MS/MS analysis. Their MS/MS spectra (Figure 10) indicated that the compounds were in fact piceasides and not other compounds with the same molecular mass (Table 1). According to the MS and UV spectra in Figure 10, the phenol called P-68 was one of piceaside G or H and the phenol called P-63 was piceaside C or D. Comparison of the MS/MS spectra showed peaks corresponding to the stilbene monomers in Figure 10B but not in Figure 10E. This could be due to the stability of the dihydrofuran ring of piceaside C and D in comparison with the dihydro-1,4-dioxin ring in piceaside G and H (Li et al. 2008a).

![Figure 9](https://example.com/figure9.png)

**Figure 9.** Structures of stilbene dimers found in Norway spruce root bark.
Figure 10. Spectra of phenols P-68 (A-C) and P-63 (D-F). The top row (A and D) shows full scan ESI-MS spectra, the second row (B and E) shows MS/MS-spectra of the main peak in the full scan spectra (809 in A and 823 in D) and the bottom row (C and F) shows UV spectra.

Table 1. Explanation of ion fragments from MS/MS-spectra in Figure 10 B and E.

<table>
<thead>
<tr>
<th>[M-H]- MS/MS fragments</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>809 647</td>
<td>Loss of glucose (809-162)</td>
</tr>
<tr>
<td>485</td>
<td>Loss of two glucose groups (809-324)</td>
</tr>
<tr>
<td>405</td>
<td>Astringin</td>
</tr>
<tr>
<td>403</td>
<td>Split product [M-H-astringin (406)]-</td>
</tr>
<tr>
<td>243</td>
<td>Piceatannol, astringin aglycone</td>
</tr>
<tr>
<td>241</td>
<td>Aglycone of split product 403</td>
</tr>
<tr>
<td>823 661</td>
<td>Loss of glucose (823-162)</td>
</tr>
<tr>
<td>499</td>
<td>Loss of two glucose groups (823-324)</td>
</tr>
<tr>
<td>243</td>
<td>Isorhapontigenin, aglycone monomer</td>
</tr>
</tbody>
</table>
3. Differences in chemistry between mini seedlings and conventional seedlings

Large pine weevils, *Hylobius abietis* (L.), feed from the bark and phloem of plants and are a great problem to the Swedish forestry because they damage and kill newly planted seedlings of pine and spruce (Långström and Day 2004). The weevils fly to clear-cuttings guided by volatiles emitted from freshly cut stumps, where they lay their eggs. The damage is caused by their feeding on newly planted seedlings. Approximately 350 million conifer plants are planted in Sweden each year (Loman 2010) and of them over 40% are treated with insecticides (www2 2009). Without insecticides, the estimated costs of pine weevil damage would be 400 million SEK higher every year (Thuresson et al. 2003).

Conifer reforestation is normally performed with one to two year 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 are attacked less frequently by the large pine weevil than the conventional seedlings (II). The mini seedlings are located by the weevils to the same extent as the conventional ones (Mitsell 2005), but they do not trigger the same feeding behaviour. 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).

![Figure 11. Structures of α-pinene and limonene. The enantiomers are perceived differently by the large pine weevil.](image)

The large pine weevils have several antennal odour receptors which respond selectively to a large number of compounds (Mustaparta 1975). The pine weevils have one receptor type which is selectively more tuned to the (+)-enantiomer of α-pinene and another receptor type which responds more to
(4S)-(−)-limonene than to their enantiomeric counterparts (Figure 11) (Wibe et al. 1998; Wibe and Mustaparta 1996). (4S)-(−)-Limonene is known to repel the large pine weevil (Nordlander 1990), and both enantiomers of α-pinene are attractants. Therefore, the enantiomeric composition of these compounds in the seedling emissions may affect the weevils and is important to measure.

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

1. Do conventional seedlings emit larger quantities 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?

3.1 Differences in volatiles from unwounded mini seedlings and conventional seedlings

It could be expected that 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 ambiguous. Even though the average emissions from mini seedlings were lower than the average emissions from conventional seedlings, the differences between the two seedling ages were not conclusive. This was due to the larger variation in emissions from the older seedlings. From some conventional seedlings we collected up to three or even seven times more terpenes than from the mini seedlings, but there were also seedlings from which we collected the same amounts as from mini seedlings, despite the much larger size and needle mass of the older plants.

What differed between the plants were the qualitative compositions of the volatile blends. The compounds emitted by the spruce plants were categorised 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 on average constituted 75% of the blend (Figure 12). Sesquiterpenes did not make up such a large fraction of the mini seedling emissions, instead a
more even distribution of compounds between the three terpene classes was observed.

![Graph showing relative amounts of volatiles](image)

**Figure 12.** Relative amounts of volatiles released by unwounded mini seedlings (n = 8) and conventional seedlings (n = 7). GLV: green leaf volatiles, MT: monoterpenes, MT-O: oxygenated monoterpenes and SqT: sesquiterpenes. No GLVs were observed in the headspace of unwounded seedlings. The total emissions of each plant were normalised to 100%, error bars denote standard errors.

Dividing the compounds into groups was helpful for indicating differences in the biosynthesis and emission between the plant age groups. But present knowledge of the odour perception and preferences of the pine weevil also had to be considered to find explanations to the lower frequency of pine weevil attacks on mini seedlings. (4S)-(−)-Limonene, a repellent of the large pine weevil, was the major compound emitted by the unwounded mini seedlings and constituted on average 16% of the volatile blend. Its enantiomeric counterpart (4R)-(+)−limonene was also present, 4% of the total amount of terpenes emitted (er∗ = 80:20, S:R). These compounds were also present in the blend of conventional seedlings, (4S)-(−)-limonene making up 4% and (4R)-(+)−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 (on average 1.2% of the compounds emitted) and the enantiomeric composition could be determined for four of the plants (minimum amount for enantiomeric analyses was 1 ng). The composition was on average 65% (1S,5S)-(−)-α-pinene and 35% (1R,5R)-(+)−α-pinene.

The differences in emissions of unwounded plants might explain the larger attractiveness of the conventional seedlings to the large pine weevil. The repellent (4S)-(−)-limonene was the most dominant compound in the volatiles of both mini and conventional unwounded seedlings, but represented a larger

*Enantiomeric ratio (er) is “the ratio of the percentage of one enantiomer in a mixture of that of the other”, according to the definition of IUPAC (1996).
proportion of the mini seedlings’ volatiles. The older plants seemed to emit a larger total amount of volatiles and in addition, the volatiles contained the attractive enantiomers of $\alpha$-pinene.

3.2 Differences in volatiles from wounded mini seedlings and conventional seedlings

Large pine weevils are more attracted to wounded than unwounded spruce plants (Nordlander 1991; Tilles et al. 1986). 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 by the needles of unwounded plants (Miller et al. 2005).

In order to investigate the change in terpene emission upon wounding, two strategies were used (A and B).

A) To see if wounding would change the enantiomer composition of limonene and $\alpha$-pinene, the phloem of the seedlings previously analysed in unwounded conditions was pierced with a needle and the volatiles were collected with SPME. Since large amounts were emitted, the volatiles were only sampled over a ten minute period. This meant that the volatiles collected were mainly the terpenes passively emitted from the fresh wound.

B) 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; thereafter the volatiles were collected with SPME for a 15-minute period and analysed with GC-MS.

As can be seen in Figure 13, the relative composition of the phloem emissions from the two seedling ages differed significantly (Monte Carlo test, $p = 0.0001$). The phloem from mini seedlings only emitted small quantities. Their emissions mainly consisted of two compounds, the green leaf volatiles ($3Z$)-hexen-1-ol and ($3Z$)-hexenal (Figure 14), together contributing on average 86% of the volatile blend. The phloem of the conventional plants, on the other hand, had a large emission, both in amount emitted and in the number of compounds. The main compound class emitted was monoterpenes, of these the two enantiomers of $\alpha$-pinene made up 38% and limonene 3%.

The enantiomeric separation of $\alpha$-pinene and limonene from the headspace of wounded conventional seedlings did not show any differences in enantiomeric composition pre or post wounding (t-test: $\alpha$-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, the emission of both limonene and $\alpha$-pinene increased but to different extents. The attractive $\alpha$-
pinenes increased from being a small part of the volatile blend to being the most dominant compounds (38 %).

Figure 13. Relative amounts of volatiles emitted by detached phloem pieces from mini seedlings (n = 11) and conventional seedlings (n = 9). There was a significant difference in composition of the emission from the phloem of the two plant types. GLV: green leaf volatiles, MT: monoterpenes, MT-O: oxygenated monoterpenes and SqT: sesquiterpenes. The emissions of each plant were normalised to 100%, error bars denote standard errors.

Figure 14. Green leaf volatiles emitted by detached phloem pieces from mini seedlings.

Previously observed increased attraction of the large pine weevil to wounded plants (Nordlander 1991; Tilles et al. 1986) can be explained by the large increase of terpenes released from the conventional seedlings observed in this study. In particular, the large increase of both enantiomers of α-pinene compared 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 for 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 against several bark beetle species (Zhang and Schlyter 2004).

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 by the
large pine weevil (Thorsén et al. 2001). In addition, naturally regenerated seedlings can withstand 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, which should increase their vigour and resistance, when compared with conventional seedlings. Variations may exist in the age at which seedlings will start to produce terpenes. Seedlings with a delayed terpene production may be of interest to conifer tree breeding programs.
4. Stress induced volatiles

The volatile profile of spruce plants differ between ages and individual plants. In addition, the emission changes when a plant is under stress. Factors eliciting 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, such as insect feeding, draught and oviposition, affect the volatile emission of conifers (Blande et al. 2009; Kännaste et al. 2009; Miller et al. 2005; Mumm et al. 2003; Ormeno et al. 2007; Priemé et al. 2000) while others (e.g. ozone stress) do not (Lindskog and Potter 1995).

When a stress response is elicited, a chain of reactions takes place in the conifer which finally leads to an altered biosynthesis of, for example, 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 induces stress reactions 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 relations 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 to conifer tree breeding programs.

I have studied the volatile response of Norway spruce plants to synthetic and biotic stress elicitors. The research questions addressed in the following chapter are:

1. How is the volatile emission of Norway spruce affected by spruce shoot aphid feeding?
2. Which chemicals in the volatile blend are emitted by the plants and which are emitted by the aphids?
3. Is it possible to isolate and collect the emissions of a single needle in vivo?
4. Is the volatile response to MeJA and the spruce spinning mite local or systemic?

The chapter ends with a short discussion on the magnitude of single needle emissions and how spruce emissions may affect the atmosphere.
4.1 Release of volatiles in response to aphid infestation and identification of aphid pheromone components (I)

Aphids locate their hosts both through volatiles as well as through chemical cues found when penetrating the plant (Powell and Hardie 2001). The spruce shoot aphid, *Cinara pilicornis*, feeds from the phloem of Norway spruce by penetrating the bark with its stylet and getting nourishment from the sap (Figure 15). The species is seldom considered a pest and it has mainly received interest as a biological predictor of air pollution (Holopainen et al. 1995; Holopainen and Kossi 1998; Holopainen et al. 1993; Kainulainen et al. 1993; Viskari et al. 2000a; Viskari et al. 2000b). We studied the chemicals released by the aphids and the induction of volatiles from Norway spruce plants.

![Figure 15. Images of spruce shoot aphids feeding on a Norway spruce plant.](image)

4.1.1 Volatile emission induced by the spruce shoot aphid

Norway spruce plants were either naturally or artificially infested with *C. pilicornis* and their emissions were compared with uninfested control plants. Within a week, infestation resulted in elevated emissions of the stress related compounds methyl salicylate, (6E)-β-farnesene and (3E,6E)-α-farnesene (Figure 16). In contrast to the farnesenes, methyl salicylate was absent in the volatiles from control plants and was thus the compound with the highest increase after infestation with aphids.
**Figure 16.** Chromatograms of emissions from plant artificially infested with *C. pilicornis*, before, one week and two weeks after infestation. The intensity of each chromatogram is normalised to the strongest one; i.e. 2 weeks after infestation. MeSA: methyl salicylate, EβF: (6E)-β-farnesene, EEαF: (3E,6E)-α-farnesene. Structures of compounds are found in Appendix I.

**4.1.2 Volatiles emitted by the spruce shoot aphid**

In addition to the stress-induced volatiles, *cis-trans*-nepetalactone, *cis-trans*-nepetalactol and citronellol were present in the odour blend when infested plants were investigated during the autumn (October-November). During other periods of the year, these compounds were absent and it was shown that they were not emitted by the plants but by the aphids (see Figure 3 in I). Figure 17 shows the proportions of aphid emitted compounds collected. *cis-trans*-Nepetalactone was the major component, constituting almost 90% of the volatiles.

**Figure 17.** Volatiles collected from headspace of spruce shoot aphid *C. pilicornis*. Five replicates with six aphids in each replicate. Error bars show standard deviation. Structures of compounds are found in Appendix I.
cis-trans-Nepeatlactone and cis-trans-nepetalactol function as sex pheromones in several aphid species (Birkett and Pickett 2003; Dawson et al. 1990). Nevertheless, they have not been described in species of the genus Cinara before. Although no behavioural studies have been performed on the spruce shoot aphid and its response to cis-trans-nepetalactone or the alcohol, one may conclude that it is probable that the compounds have a pheromone function in this aphid species. The discovery that the compounds were only emitted during the autumn period, and not during other seasons, supports this hypothesis.

Citronellol, on the other hand, has not shown any behavioural or electrophysiological effects on the aphid species it has been tested upon. Instead it has been suggested that it is a precursor to nepetalactone biosynthesised by aphids (Dawson et al. 1996).

4.1.3 Possible effects of spruce volatiles on aphids

Although plants can gain resistance by their volatile response (Beale et al. 2006; Gibson and Pickett 1983) many aphid species have obtained the ability to deal with or even use their host’s volatiles. Jackson et al. (1996) studied four aphid species living on Picea sitchensis (Bong) and found that the tolerance to myrcene and piperitone varied according to the doses found at the preferred feeding site of the aphids; each species had developed higher tolerance to the monoterpene they encountered in highest doses. C. pillicornis had the highest tolerance to myrcene and lowest tolerance to piperitone. Both these compounds were present in the volatiles of the naturally infested clone grafts but at lower amounts than from the uninfested control plant.

(6E)-β-Farnesene was one of the major compounds released by aphid infested plants and while many aphid species use this compound as an alarm pheromone (Pickett and Griffiths 1980; Xiangyu et al. 2002) behavioural tests with two other Cinara species detected no alarm behaviour upon exposure to it (Xiangyu et al. 2002).

No (6E)-farnesene was found in aphid emissions, not even when the aphid were manipulated to excrete cuculliar drops (I). It seems likely that the conifer feeding Cinara genus do not use (6E)-β-farnesene as an alarm pheromone and it is interesting to speculate whether this might be the cause of coevolution between conifers and their associated aphids. (6E)-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). However, there are also examples of aphids that use other plant volatiles as cues to distinguish the sources of (6E)-β-farnesene emission. Dawson et al. (1984) found that the
effect of 6E-β-farnesene as an alarm pheromone was inhibited if (1R,9S)-(−)-β-caryophyllene or volatiles from uninfested hop plants were released together with (6E)-β-farnesene.

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). Methyl salicylate was the compound most induced by artificial spruce shoot aphid infestation on Norway spruce seedlings (I) and it has recently been shown to increase the response of male aphids of the species Rhopalosiphum padi and Phorodon humuli to their respective 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 methyl salicylate acts as antifeedant for the large pine weevil (Borg-Karlson, personal communication) as well as inhibiting the attraction of the weevils to odours from Norway spruce twigs (Kännaste et al. 2009).

4.2 Localisation of volatile response: headspace analysis of individual needles in vivo (III)

A stress elicitor gives rise to a reaction at the site of attack and 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).

MeJA application on the stem elicits a short distance systemic anatomical response in Norway spruce (Franceschi et al. 2002). Traumatic resin ducts and PP-cells are formed within the stem. Those formed above the treated area have features indicating a later and weaker induction of formation than the ones at the MeJA-treated site.

A volatile systemic response of Norway spruce to the large pine weevil is described by Blande et al. (2009). Another example among conifers is the indirect systemic volatile defence of Pinus sylvestris against the pine saw fly, Diprion pini (Hilker et al. 2002; Mumm et al. 2003). Systemic effects in the volatile responses of conifers to MeJA have not been studied in previous investigations because the MeJA-treatments were accomplished by spraying whole plants with MeJA solution (Martin et al. 2003; Miller et al. 2005). Application on the lowest part of the stem with a soft brush gives a similar volatile response as spraying the whole plant (Pettersson 2007) and this technique can be used to study systemic volatile responses.

The spruce spinning mite Oligonychus ununguis is a mite species that feeds on Norway spruce needles. They suck their nourishment from the parenchyma
cells and cause yellow spots and browning of needles. Their effect on the volatile emission from Norway spruce is described by Kännaste et al. (2009). (3E,6E)-α-Farnesene and methyl salicylate are the main compounds emitted, together with the less abundant (6E)-β-farnesene and benzoic acid. The farnesenes are emitted as a response to various stress elicitors (I; III; Blande et al. 2009; Kännaste et al. 2009; Martin et al. 2003; Pettersson 2007) and are suitable targets for monitoring stress reactions of Norway spruce.

The single needle method was developed to isolate and collect the volatiles of individual needles in vivo. The method was used to study the localisation of the response to MeJA application and mite infestation.

4.2.1 Headspace analysis of individual needles in vivo
In order 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, which isolated its headspace. An SPME was placed into the glass tube and the fibre was exposed for 22 hours to collect the volatiles from the needle. This setup made it possible to follow the change in volatile emission upon stress for one separate needle.

![Chromatogram areas of seven compounds, typical in the headspace of spruce, collected with SPME (PDMS/DVB) in the single needle setup (III). 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. Structures of compounds are found in Appendix I.](image)

Figure 18. Chromatogram areas of seven compounds, typical in the headspace of spruce, collected with SPME (PDMS/DVB) in the single needle setup (III). 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. Structures of compounds are found in Appendix I.

Competition effects can occur when porous SPME fibres are used with long adsorption times. Plants do not only emit a multitude of volatile organic compounds (VOCs) but during respiration water is also emitted and can interfere with the SPME. Possible competition effects of other terpenes or water on the adsorption of (6E)-β-farnesene were therefore investigated. A
setup simulating the single needle one was used. No significant differences in adsorbed amounts could be detected, neither for the compound in a terpene mixture with all compounds of equal concentration, nor for $(6E)$-$\beta$-farnesene in the terpene mixture with a large excess of water (Figure 18). Two compounds were collected in significantly less amounts when water was present; $\alpha$-pinene ($p = 0.005$) and limonene ($p = 0.017$, t-test assuming unequal variance).

4.2.2 Local emission of induced volatiles
The single needle setup was used together with a GC-MS program, optimised for the fast detection of farnesenes, to study stress induced volatile reactions on the scale of individual needles. Two-year old Norway spruce seedlings (conventional seedlings, for planting in reforestation) were applied with MeJA (1:100) on the five lowest centimetres of the stem by a soft brush. Two needles on the treated areas and two needles growing above (one and two centimetres up, respectively) were analysed using the single needle setup. Needles growing on MeJA-treated areas emitted $(6E)$-$\beta$-farnesene, $(3E,6E)$-$\alpha$-farnesene, $(E)$-$\alpha$-bisabolene and linalool. These compounds were mostly absent in the headspace of the needles growing above the MeJA-treated area, with only one of the needles emitting small amounts (Figure 19A and B). The clear difference indicated a highly localised response immediately after MeJA-application.

Figure 19. Chromatograms of head space-SPME-GCMS analyses of A) Needle growing on MeJA-treated area, B) Needle on the same plant as A, but growing above MeJA-treated area, C) Needle with one feeding spruce spider mite, D) Needle on the same plant as C, but growing on a branch without mites. The intensity of each chromatogram is normalised to the strongest one; i.e. C. MeJA: methyl jasmonate. E$\beta$F: $(6E)$-$\beta$-farnesene, EEnF: $(3E,6E)$-$\alpha$-farnesene, E$\alpha$B: $(E)$-$\alpha$-bisabolene. Structures of compounds are found in Appendix I.
A similar pattern was found in the volatiles from needles exposed to feeding spider mites (Figure 19C and D). Emissions on needles growing on infested and uninfested areas of the same plants were investigated. The main compounds found were $(3E,6E)$-α-farnesene together with $(6E)$-β-farnesene and $(E)$-α-bisabolene, substances absent in the headspace of uninfested needles.

These results indicate that both MeJA and infestation of spruce spinning mites give rise to a local volatile response in Norway spruce plants. Nevertheless, a systemic response should still be considered.

The systemic response needs time to develop; the signal should be formed and transferred through the plant and the biosynthetic reaction induced. The farnesene response to MeJA follows a diurnal cycle that peaks the first 24-48 hours after application (Martin et al. 2003; Pettersson 2007). The SPME-collections in this study were made during this period and this is probably too short a time for the systemic response to develop. Repeated MeJA applications may provoke a systemic volatile reaction.

Previous studies detected a systemic response from twigs, which were exposed to insects feeding or egg laying (Blande et al. 2009; Hilker et al. 2002). In this study, plants with a natural mite infestation were used and the needles were chosen from different twigs; one infested and one which the mites had not yet reached. It is possible that a systemic volatile reaction localised to the infected twig could be found under controlled conditions. For a parasite or predator, it is advantageous to be guided as close as possible to its host or food source. Consequently, it can be more beneficial for a plant or a tree to respond to an aggressor with a local or a small scale systemic reaction than with a large systemic reaction involving more parts of the tree.

The single needle method makes it possible to detect and follow an induced volatile reaction at the scale of individual needles. If the actions of aggressors within a branch, e.g. fungal pathogens, give rise to altered needle emissions, the single needle setup would be a non-invasive method to monitor the progress of the aggressor. The single needle method also gives us a tool to estimate the magnitude of stress induced emissions; emissions that do not only affect other organisms but may also contribute to the atmospheric chemistry.

4.3 Spruce emission contributes to volatile organic compounds in the atmosphere

The VOCs emitted by spruces do not only act as semiochemicals in interactions with insects, but may also have an impact on atmospheric characteristics. Common spruce emissions such as monoterpenes and sesquiterpenes, including the stress induced farnesenes, linalool and the phenol methyl salicylate, are involved in different processes in the atmosphere where they undergo oxidation.
processes to unsaturated ketones and acids, and form secondary aerosols (Atkinson and Arey 2003; Goldstein and Galbally 2007; Helmig et al. 2006; Joutsensaari et al. 2005; Karl et al. 2008; Lee et al. 2006). These can in an indirect way affect the climate and thus the conditions for the ecological community.

Since the contribution of biogenic VOCs to atmospheric mechanisms is of high importance, various models have been developed to simulate and project VOC emissions (Grote and Niinemets 2008). One of the challenges is to acquire emission data to build these models on. The semi-volatile and highly reactive sesquiterpenes are difficult to measure with traditional air sampling methods and SPME has been suggested to be a suitable technique to provide sesquiterpene emission data (Baker and Sinnott 2009; Bouvier-Brown et al. 2007). One technical consideration with SPME is the previously discussed competition effects that can occur with the use of porous fibres, such as the PDMS/DVB fibre used in this work.

In the single needle study the largest quantities of (6E)-β-farnesene, (3E,6E)-α-farnesene and (E)-α-bisabolene collected from a single needle summed up to 60 ng/22 h corresponding to on average 4 ngC gDW⁻¹ h⁻¹† (unpublished data). We did not calibrate for the sesquiterpenes in air samples but the results from the competition study indicate that it may be as little as 5-10 % of the available amount that is collected. That would lead to a needle emission of 0.04-0.08 μgC gDW⁻¹ h⁻¹ which is within the range (0.02-3.325 μgC gDW⁻¹ h⁻¹) of previously reported emission data of sesquiterpenes from ponderosa pine (Baker and Sinnott 2009; Bouvier-Brown et al. 2007; Helmig et al. 2007).

It is important for atmospheric modellers to take into account the variation and alterations of VOC emissions from plants due to stress, e.g. infestation of insects (Blande et al. 2009; Duhl et al. 2008; Joó et al. 2009). Our results from the single needle experiments imply that the emissions can differ tremendously between needles on different parts of a spruce. This is an aspect that should be considered when up-scaling emission data. It should also be acknowledged that the diversity in units in reports of plant emissions among chemical ecologists and other researchers cause problems for atmospheric modellers (Duhl et al. 2008). On the whole, atmospheric chemists would benefit from further knowledge on plant emissions.

† ngC gDW⁻¹ h⁻¹, ng of carbon (ngC) per g dry weight (gDW) and hour (h).
5. Responses to inoculation with Heterobasidion annosum

The pathogenic fungi of Heterobasidion annosum (Fr.) Bref. sensu latu (s.l.) cause butt rot to several species of conifers and approximately 15% of the spruce in Sweden show symptoms of infection (Witzell et al. 2009). The decayed wood results in huge economic losses to the forestry industry in northern Europe (Woodward et al. 1998). The losses in Sweden alone are estimated to 0.5-1 billion SEK annually (Witzell et al. 2009).

In Europe, there are three species of Heterobasidion. H. annosum (Fr.) Bref. sensu stricto (s.s.) (formerly known as the P-type) which mainly attack pines but also other tree species, and are found in pine forests all over Europe (Niemelä and Korhonen 1998). The more host specific H. parviporum Niemelä & Korhonen (formerly S-type) which mainly colonise P. abies and are found in eastern and northern Europe and H. abietinum Niemelä & Korhonen (formerly F-type) which are established in southern Europe and mainly infect species of fir (Abies spp.) (Niemelä and Korhonen 1998).

The establishment of Heterobasidion in a new conifer stand is commonly caused by spores infecting stumps or fresh wounds. Then, if the fungi manage to establish itself, the infection can spread through root contact to other trees (Redfern and Stenlid 1998). However, even in heavily infected Norway spruce stands there are a proportion of trees (at least 5%) that are uninfected (Delatour et al. 1998). There are studies that indicate that this resistance, or lower susceptibility, is caused by genetically determined host characteristics (Arnerup et al. 2010; Swedjemark and Karlsson 2004; Swedjemark and Karlsson 2006; Swedjemark and Stenlid 1997; Swedjemark et al. 1998; von Weissenberg 1975) but the cause of this inherent ability is unknown. One theory is that differences in host chemistry and the induced chemical defence could play an important role.

In recent years transcript profiling has been used to gain insight into the defence system of conifers. The combination of transcript profiling and chemical characterisation have shed light on the role of terpenes in spruce defence against insects (Martin et al. 2004; Ralph et al. 2006; Zulak and Bohlmann 2010), but so far the interactions with fungi have been less well investigated. Adomas et al. (2007) investigated the response of Pinus sylvestris to inoculation with H. annosum s.s. and found that many of the genes up-regulated after inoculation were coding for defence related proteins or for enzymes involved in the phenylpropanoid pathway. Arnerup and co-workers
got similar results from a study on Norway spruce (unpublished). After *H. parviporum* inoculation they found indications that carbon was redirected from protein synthesis to the shikimic acid and phenylpropanoid pathways, presumably leading to increased production of phenols in the form of lignin, stilbenes and flavonoids. But there is still much to investigate in order to gain a thorough understanding of the defence mechanisms of Norway spruce.

We have studied both the transcriptional and chemical response of Norway spruce clones, with known susceptibility to natural infection with *Heterobasidion* spp., to inoculation with *H. annosum s.s.* with the aim of answering the following research questions:

1. Are there any chemical markers of resistance in the constitutive chemical composition, which can indicate if a tree is more or less susceptible to *Heterobasidion* infection?
2. How do trees respond to fungi inoculation; with a focus on terpene chemistry, phenol chemistry and their transcriptional regulation?

### 5.1 Clone material, susceptibility and necrosis length

The trees used in this study were part of a Swedish regional clonal forestry programme at SkogForsk (Karlsson and Högberg 1998). In a previous study the clones showed genetic variation in susceptibility to natural infections of *Heterobasidion* spp. (Karlsson and Swedjemark 2006) and thus formed a unique material for studies relating susceptibility to chemical traits. Nine clones, representing the most and least susceptible types, were chosen for the inoculations. Three trees of each clone were included in the study and on each tree two roots were treated. One was inoculated with *H. annosum s.s.* and the other was wounded, i.e. mock-inoculated by a sterile wooden plug. Samples were collected from each root at the time of inoculation, 5 days post inoculation (d.p.i), 15 d.p.i. and 28 d.p.i. More details on the experimental setup can be found in IV.

The necrosis lengths were measured at 44 d.p.i. and in Figure 20 average values for each clone are presented together with the clone’s visual rot frequency from natural infestation. No differences in necrosis lesion length between more and less susceptible clones were found (single factor ANOVA, wounded $p = 0.29$, inoculated $p = 0.39$). But the lesion lengths were longer for inoculated roots than for wounded roots (single factor ANOVA, $p = 0.0003$). This indicates that the fungi inoculation succeeded and elicited a stronger response than wounding alone. The results also suggested that necrosis length of inoculations may not be a suitable measurement of susceptibility but rather as a measure of the tree response to the fungi.
Figure 20. Necrotic lesion lengths (mm) measured 44 days post inoculation (d.p.i), average values for each clone with standard bars representing standard errors. The clone numbers are presented on the x-axis together with the visual rot frequency for each clone as found by Karlsson and Swedjemark (2006).

Four clones (2405, 7398, 3178 and 3340) were chosen for full transcriptome analysis with 454-sequencing and chemical characterisation by GC-MS and LC-MS. Samples from the other clones were used to confirm the occurrence of interesting compounds or transcriptome patterns. The less susceptible clones 2405 and 7398 did not show complete resistance to the pathogen (Figure 20) but to simplify the discussion they will henceforth be referred to as resistant clones and clones 3178 and 3340 will be referred to as susceptible clones.

5.2 Terpene constitutive composition and induced response

Since terpenes are such a prominent part of conifer defence, they have been extensively studied, including their interaction with *Heterobasidion*. Inoculation with *H. annosum* causes formation of more and bigger traumatic resin ducts in Norway spruce stem tissues than wounding does (Krekling et al. 2004), and several studies show an increase of terpene amounts after inoculation (Asiegbu et al. 1998 and references therein). The general view is that while monoterpenes have fungitoxic effects the role of resin acids is to act as mechanical barriers to the fungi (Asiegbu et al. 1998). Although many *in vitro* studies have been performed on the antifungal effects of terpenes, the results are often conflicting (Asiegbu et al. 1998 and references therein). This is probably caused by the use of different bioassay methods, different strains or species of fungi and different terpene concentrations tested.
Two recent studies have investigated the induction of terpenes in the defence reaction to \textit{H. annosum}. Woodward et al. (2007) looked for potential markers of relative susceptibility in Sitka spruce (\textit{Picea sitchensis}). They found higher contents of (1\textit{R},5\textit{R})-(+)-\textalpha-pinene, (1\textit{S},5\textit{S})-(−)-\textbeta-pinene and one unknown terpene in clones less susceptible to \textit{Heterobasidion} infection than in more susceptible clones. In that study 3-carene and two unidentified terpenes increased more in less susceptible trees, after inoculation, than in more susceptible trees (Woodward 2007). Zamponi et al. (2007) reported a higher increase of (1\textit{S},5\textit{S})-(−)-\textalpha-pinene, (1\textit{R},5\textit{R})-(+)-\textalpha-pinene and 3-carene after inoculation of \textit{Picea abies} branches with \textit{Heterobasidion} spp. compared with samples from wounded branches.

\textbf{5.2.1 Constitutive differences between resistant and susceptible clones}

We found no clear patterns indicating a relationship between terpene composition and susceptibility. A PCA (Figure 21) showed that the first PC explained 57\% of the variation and was mainly associated with terpene concentration. Strong samples could be found to the right in the plot together with most variables. The second PC had a tendency to separate resistant and susceptible clones but only explained 15\% of the variation, and a Monte Carlo test did not show significant differences between the two groups (\textit{p} = 0.059). The only terpene exclusively found in resistant trees was \textalpha-longipinene (T-10).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure21.png}
\caption{PCA biplot of constitutive terpene samples based on chromatogram areas divided by internal standard area and sample dry weight. Grey samples denote susceptible clones. Terpene numbers (T-#) are explained in Appendix II, Table A1.}
\end{figure}
5.2.2 Response to inoculation
The total amounts of terpenes increased after inoculation and wounding (Figure 22). The increase was stronger for inoculated than wounded roots and diterpenes increased more than monoterpenes.

![Figure 22](chart.png)

**Figure 22.** Total terpene increase after inoculation and wounding based on chromatogram areas divided by internal standard area (IS) and sample dry weight (DW). IS eq.: Internal standard equivalents. Error bars show standard deviations.

A PCA based on relative terpene composition (Figure 23) showed that samples tended to group according to clone identity, but not according to susceptibility. No consistent patterns in arrangement of the samples according to treatment or time after inoculation were found.

The effect of inoculation was studied further for three monoterpenes: \((1S,6R)-(+)-3\)-carene, \((4R)-(+)\)-limonene and \((4S)(-)-limonene. The general pattern of a larger increase after inoculation and wounding was confirmed for \((1S,6R)-(+)\)-3-carene by expanding the data set with the five clones not included in the transcriptome and screening chemical analyses (Figure 24). Significant differences between treatments were found at 28 d.p.i. (paired t-test, \(p = 0.008\)), the increase from day 0 was significant for inoculated samples (\(p = 0.008\)) but not wounded samples (\(p = 0.06\)). No significant differences could be found between resistant and susceptible clones. Hence \((1S,6R)-(+)\)-3-carene could serve as a marker for fungal infection but not for susceptibility against *H. annosum*. 


Figure 23. PCA biplot of terpene composition data (the total areas of each chromatogram normalised to 100%). Odd numbered samples correspond to inoculated roots and even numbered to wounded roots. Resistant clones 2405 and 7398 are coloured with a yellow-red scale while the susceptible clones 3178 and 3340 are coloured with a green-blue scale. The percentages of the axes states how much of the variation the corresponding PC explain. Terpene numbers (T-#) are explained in Appendix II, table A1.

Figure 24. Induction of (1S,6R)-(+)-3-carene after inoculation and wounding. Chromatogram areas divided with internal standard (IS) area and dry weight (DW) of sample, IS eq.: internal standard equivalents. Data from 16 trees (2 roots per tree, one for each treatment) corresponding to 9 clones. Error bars show standard deviations.

3-Carene has been a compound of interest in previous studies of fungi-spruce interactions and our results are in agreement with earlier findings in Norway spruce. Zamponi et al. (2007) noted a relative increase of 3-carene after inoculation with any of the three European *Heterobasidion* spp. They
concluded that it may be an important factor in the response to *Heterobasidion s.l.* infection since it was the only monoterpene which was strongly induced by all three *Heterobasidion* species. 3-Carene induction seems to be a general response to fungal inoculation because it is also induced after inoculation with other fungal species such as *Ceratocystis polonica* (Zhao et al. 2010). As mentioned in the beginning of this section, Woodward et al. (2007) discovered differences in induction of 3-carene between more and less susceptible Sitka spruce plants, where the relative increase of 3-carene after wounding or inoculation were only significant for the less susceptible clones. In our study there were indications for a stronger (1S,6R)-(+-)3-carene induction in resistant clones compared with susceptible ones, but the differences were not significant.

Clone 7398 seemed to have a stronger induced limonene production than clone 3178 28 days after both wounding and inoculation. However, the clones responded with different enantiomeric patterns (Figure 25). Before treatment (4R)-(+-)limonene was the dominant enantiomer of both clones but clone 3178 had a stronger induction of (4S)-(--)limonene than (4R)-(+-)limonene, resulting in a different enantiomeric ratio. This could be an indication of stronger induction of (--)limonene synthase (PaTPS-Lim) in 3178 than 7398. Woodward et al (2007) found a higher relative abundance of (4S)-(--)limonene in constitutive resin of more susceptible Sitka spruce clones compared with less susceptible clones. Our results did not indicate any differences in constitutive amounts and to draw conclusions of connection to susceptibility more clones need to be investigated.

![Figure 25. Induction of limonene after inoculation (I) and wounding (W) of clone 7398 and 3178. Chromatogram areas divided with internal standard (IS) area and dry weight (DW) of sample, IS eq.: internal standard equivalents. Enantiomeric ratios (4S:4R) are presented above the columns and error bars show standard deviations.](image-url)
5.2.3 Comparison with gene regulation
The PCA of terpene contents (Figure 23) did not show patterns corresponding to treatment or susceptibility. This corresponds with the results from clustering analysis of TPS-like genes (IV). The hypothesis of stronger up-regulation of PaTPS-Lim at 28 d.p.i. in clone 3178 compared with clone 7398 was based on the observation of changed enantiomeric ratio of limonene in clone 3178 (Figure 25). However, it could not be confirmed by the regulation patterns of PaTPS-Lim in our data set (IV). In addition, the different levels of (1S,6R)-(+)3-carene between inoculated and wounded samples 28 d.p.i. (Figure 24) had no correlation in the regulation of PaTPS-Car like genes (IV).

Martin et al. (2004) reported that expression levels of TPS-genes match the production of monoterpenes in Norway spruce, but expression levels are not the only piece in the puzzle. The enzymatic activities were not investigated in our study and this link between gene expression and metabolite products may possible explain the discrepancies between chemical and transcriptional results. It should also be noted that the limonene and gene expression results were based on data from two clones (IV), while the results of (1S,6R)-(+)3-carene induction were based on the extended data-set of all nine clones.

5.2.4 Comparison with other studies
When comparing tree chemistry responses from different inoculation studies it is important to pay attention to the details of the conditions under which the studies have been conducted. Different conifer species posses differences in biosynthesis and storage of defence metabolites (Franceschi et al. 2005), and terpene and phenol content of conifers are known to vary between tissues, ages and season (Hakola et al. 2006; Lindberg et al. 1992; Persson et al. 1993).

Comparison of three recent studies (IV; Woodward et al. 2007; Zamponi et al. 2007) showed that although all studies investigated the chemical effects of *H. annosum* inoculations on spruce, the conditions in which they were conducted differed (Table 2). The Woodward study was performed on Sitka spruce and the other two on Norway spruce. The three studies inoculated different parts of the tree, used trees of different ages and were conducted at different times of the year.

In addition there are several ways to measure the resistance or susceptibility of a tree to fungi, and these also differed between the studies. Zamponi et al. (2007) studied the species specific resistance, Woodward et al. (2007) used necrosis length after inoculation as a measure of the relative susceptibility, and we used data from natural infestation in the field (IV; Karlsson and Swedjemark 2006).
All these factors may contribute to the overall picture and should be kept in mind when comparing the outcome of the studies. If the connection between the abundance of some compounds and susceptibility to infection is indirect (i.e. that both are controlled by genetics but the compounds themselves do not affect the performance of the fungus) the results may differ widely between studies. On the other hand, if the connection between compound abundance and susceptibility is a direct one, caused by antifungal properties of the compound, one could expect to find the connection even if the conditions for the studies differ. To date however, \textit{in vitro} studies of the antifungal effects of terpenes have given contradictory results.

Table 2. Comparison of plant material, inoculation conditions and seasonal conditions of three studies on spruce and \textit{H. annosum} s.s.

<table>
<thead>
<tr>
<th></th>
<th>IV</th>
<th>Woodward et al. (2007)</th>
<th>Zamponi et al. (2007)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tree species</td>
<td>\textit{P. abies}</td>
<td>\textit{P. sitchensis}</td>
<td>\textit{P. abies} and \textit{A. alba}</td>
</tr>
<tr>
<td>Tree age</td>
<td>27 yr</td>
<td>2 yr</td>
<td>15 yr</td>
</tr>
<tr>
<td>Inoculated tissue</td>
<td>Roots</td>
<td>Stems</td>
<td>Branches</td>
</tr>
<tr>
<td>Fungi</td>
<td>\textit{H. annosum}</td>
<td>\textit{H. annosum}</td>
<td>\textit{H. annosum and H. parviporum} / \textit{H. abietinum}</td>
</tr>
<tr>
<td>Season</td>
<td>August-September</td>
<td>July-August</td>
<td>November-Mars</td>
</tr>
<tr>
<td>Temperature</td>
<td>6 – 25 °C</td>
<td>15 – 25 °C</td>
<td>-6 – +13 °C</td>
</tr>
</tbody>
</table>

5.3 Phenolic constitutive composition and induced response

Phenol chemistry has been extensively studied in fungi-conifer interactions, with much attention on stilbenes. Common responses to fungal inoculation are swelling of PP-cells and a change in phenol glycosation. Upon inoculation, the stilbene glycosides decrease with a subsequent increase of the corresponding aglycones (Brignolas et al. 1995a; Franceschi et al. 2000). Structures of stilbenes can be found in Figure 26 and Appendix I.

Susceptibility of Norway spruce to \textit{C. polonica} has been associated with overall constitutive phenol composition and isorhapontigenin content in the tree (Brignolas et al. 1998; Lieutier et al. 2003), and also with the density of PP-cells and speed of defence mechanism (Nagy et al. 2004). Even though some studies on \textit{H. annosum} s.l. did not find a correlation between stilbene content and susceptibility of the host (Toscano Underwood and Pearce 1992; Witzell and Martin 2008; Woodward and Pearce 1988), Lindberg et al. (1992) found the constitutive occurrence of astringin to be negatively correlated with the depth of \textit{H. parviporum} hyphen growth in Norway spruce.
Multivariate analyses (PCA and RDA) of the relative phenol composition were used to identify abundant phenols in the bark of resistant clones, which could be potential markers of resistance. The PCA had a tendency to separate the resistant and susceptible clones on the first PC, which explained 30% of the variation in the data set (Figure 27). This trend showed that there may be a correlation between susceptibility and overall phenol composition, similar to the findings of Brignolas et al. (1998) for the susceptibility of Norway spruce to *C. polonica*. The compounds with lowest scores on the PC1 (< -0.75) were considered typical for resistant clones and can be found in Table 3.
**Figure 27.** PCA biplot of phenol samples collected day 0 based on constitutive composition (MS-chromatogram areas with the sum area of each chromatogram normalised to 100%). Grey samples denote susceptible clones. The vertical lines show where compounds of possible interest can be found (compounds contributing <-0.75 or >0.75 to PC1). Phenol numbers (P-#) are explained in Appendix II, Table A2.

**Table 3.** Compounds of higher abundance in the bark of resistant clones presented with their deprotonated molecular ion mass and characteristic UV maxima values. Compound numbers refer to the numbers in Figures 27 and 28.

<table>
<thead>
<tr>
<th>Compound No.</th>
<th>Compound</th>
<th>[M-H]$^-$</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-24</td>
<td>Unknown glucoside</td>
<td>539</td>
<td>279</td>
</tr>
<tr>
<td>P-35</td>
<td>(E)-Astringin</td>
<td>405</td>
<td>323</td>
</tr>
<tr>
<td>P-52</td>
<td>Piceaside A or B</td>
<td>809</td>
<td>329</td>
</tr>
<tr>
<td>P-66</td>
<td>Piceaside G or H</td>
<td>809</td>
<td>323</td>
</tr>
<tr>
<td>P-68</td>
<td>Piceaside G or H</td>
<td>809</td>
<td>323</td>
</tr>
<tr>
<td>P-94</td>
<td>Unknown glucoside</td>
<td>583</td>
<td>331</td>
</tr>
</tbody>
</table>

A Monte Carlo permutation of the phenol composition of the two susceptibility groups indicated that there was indeed a difference ($p = 0.003$) and RDA showed that in addition to the compounds listed in Table 3 the unknown compounds P-3 and P-7 were also typical for resistant clones (limit set to scores lower than -0.5 on axis 1). These two compounds were absent in clone 3371 and 3340 and had scores lower than -0.70 on PC1 in the PCA (Figure 27).

(E)-Astringin was one of the phenols typical to resistant clones, which was consistent with the results of Lindberg et al. (1992). Both (E)-astringin and
astringin dimers (P-52, P-66 and P-68) were typical to resistant clones. Piceasides were not measured in previous studies since they were first identified by Li et al. in 2008 and our result suggested that they may be of importance in the interaction with *Heterobasidion* spp.

### 5.3.2 Response to inoculation

Before inoculation, the phenolic pattern differed between resistant and susceptible clones but inoculation resulted in a more unified pattern, with increased amounts of less polar compounds. In a PCA based on relative phenol composition (Figure 28) the samples had a tendency to order themselves on the first PC according to time and treatment. The constitutive samples were found to the left in the plot and samples from inoculated roots 15 and 28 d.p.i. were found furthest to the right. The second PC tended to separate the constitutive samples of resistant and susceptible clones from each other, and this separation held in early phases of inoculation and wounding. However, as time after inoculation passed, the phenol composition of inoculated roots became increasingly similar between the two clone groups and the samples were found in the lower right of the plot.

**Figure 28.** PCA biplot of phenol data based on relative MS-chromatogram areas (the sum area of each chromatogram normalised to 100%). Resistant clones are coloured as yellow (d0), light orange (d5), dark orange (d15) and red (d28). Susceptible clones have the colour coding light green (d0), dark green (d5), turquoise (d15) and blue (d28). Circles denote samples of constitutive phenol composition, diamonds samples from inoculated (I) roots and squares samples from wounded (W) roots. The percentages of the axes states how much of the variation the PC explain. Phenol numbers (P-#) are explained in Appendix II, Table A2.
The total amount of stilbenes decreased after both inoculation and wounding, but the initial response in wounded roots seemed to be limited to the first five days while the decrease continued in inoculated roots (Figure 29). As stilbene glucosides decreased a concurrent increase in the corresponding aglycones was observed, which were in accordance with previous studies (Brignolas et al. 1995b; Johansson et al. 2004; Lindberg et al. 1992; Viiri et al. 1996).

Figure 29. Changes in stilbene content after inoculation and wounding represented by average MS-chromatogram areas divided with sample dry weights. Standard bars show standard errors. Diamonds denote glucose monomers, open diamonds isomers of the glucose monomer, triangles glucose dimers and square the corresponding aglycones. Astr dim: sum of piceasides A, B, G and H (dimers of two astringin molecules), IsoR/Astr dim: sum of piceasides C-F (stilbene dimers of one astringin and one isorhapontin).
The hydrolysis of the glycosidic bond has been assigned to the activity of \(\beta\)-glycosidase from either the fungus (Woodward and Pearce 1988) or the tree (Johansson and Stenlid 1985).

Different stilbenes did not seem to respond to inoculation in a similar way (Figure 29). The decreasing pattern of astringin was more similar to the decreasing pattern of stilbenes in wounded roots than to the other stilbene glucosides of inoculated roots. This could be due to differences in regulation, but also because astringin is released in the breakdown of stilbene dimers.

### 5.3.3 Comparison with gene regulation

The phenolic pattern changed upon treatment and the samples from inoculated roots got a phenol composition that differed from the other samples at 15 and 28 d.p.i. Even though the metabolite pattern consisted of the compounds produced up to that point and the transcriptome expression was a snapshot on the biochemical processes in the sample; a similar pattern could be found among gene regulations. At 15 d.p.i. the clustering of genes in the phenylpropanoid pathway separated samples according to treatment, i.e. wounding or inoculation (IV).

![Figure 30. Structures of catechin and epicatechin.](image)

Leucoanthocyanidin reductase (LAR) catalyses the synthesis of catechins from leucoanthocyanidins (see Figure 9 in IV). LAR was up-regulated at 5 d.p.i. (IV) and was followed by an increase of \((2R, 3S)-(+)-catechin\) between 5 and 15 d.p.i. Together with LAR, other genes, corresponding to enzymes in the catechin/epicatechin biosynthetic pathways, were also up-regulated (IV). Catechins and epicatechins (Figure 30) are monomers used in the polymerization reaction forming proanthocyanidins (Zhao and Dixon 2009). Proanthocyanidins (or condensed tannins) and cell wall bound phenolics have been suggested to play a role in conifer protection against beetles and pathogens (Brignolas et al. 1995a; Schmidt et al. 2005).
6. Conclusions

- A method to separate and identify the four diastereomers of nepetalactone by GC-MS has been developed.

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

- Among the volatiles produced (4S)-(−)-limonene and the green leaf compounds were possible candidates of resistance markers for mini seedlings against *Hylobius abietis*.

- Feeding of *Cinara pilicornis*, *Oligonychus ununguis* or stem application of methyl jasmonate induced volatile responses with methyl salicylate, (6E)-β-farnesene, (3E,6E)-α-farnesene and (E)-α-bisabolene the main compounds emitted.

- Volatiles were collected from live spruce shoot aphids; during short autumn periods nepetalactone, nepetalactol and citronellol were found in the headspace of the aphids.

- 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 was found to the stress elicitor methyl jasmonate and to spruce spinning mite feeding.

- Variation in natural susceptibility to *Heterobasidion* spp. infection may be connected with constitutive levels of astringin, astringin dimers (piceasides) and α-longipinene in the root bark.

- Inoculation of Norway spruce trees with *Heterobasidion annosum* s.s. elicited a general increase of terpene content and a treatment dependent phenolic response. Corresponding patterns could be found in the transcriptome.
Acknowledgements

The field of chemical ecology gives many opportunities to collaborations and I wish to express my gratitude to all who have contributed to make this work possible.

First of all, I would like to thank my supervisor Prof. Anna-Karin Borg-Karlson for accepting me as a PhD-student, for sharing your knowledge and enthusiasm, for support during all these years and for making the group of Ecological Chemistry such a stimulating and enjoyable place in which to work.

Prof. em. Tobjörn Norin, Dr. Malin Elfstrand and Nélida Gonzáles are gratefully acknowledged for valuable comments on this thesis and Douglas Jones for linguistic revision.

Prof. Rikard Unelius initiated the nepetalactone study and I am thankful for your valuable help and discussions during the work. I also wish to thank Dr. Ellen Santangelo for answering my many questions and sharing your experience in the lab.

Everyone on the mini seedling team is appreciated for a nice collaboration. Thank you for answering my biological questions and making me explain the chemistry to you. Dr. Astrid Kännaste is acknowledged for performing the enantiomeric analyses discussed in chapter 3.

To my collaborators on the Heterobasidion project: it is a pleasure to work with you! I appreciate your patience with my many questions about biochemistry, forestry and mycology and have enjoyed our time in the forest. My colleagues at KTH: Karolin Axelsson, Anders Molin and Dr. Katinka Pålsson are thankfully acknowledged for help in the lab and the field. Tao Zhao, I am grateful for our many discussions on conifer chemistry on this and other projects. Jiang Hu is acknowledged for performing the enantiomeric analyses discussed in chapter 5.

A special thanks to Annie Yart who introduced me to the analysis of phenolics in bark, provided stilbene and flavonoid reference compounds and made my short stay in France a memorable time.

Formas and KTH are gratefully acknowledged for financial support. The Aulin-Erdtman foundation, Knut and Alice Wallenberg’s foundation, the Lindau foundation, Fredrik Björn’s foundation and IFS-MISTRA for making it possible to participate in interesting and inspiring conferences and courses.
I would also like to thank:
All my collaborators on projects not mentioned in this thesis
   My present and former colleagues in the Ecological Chemistry group for
       interesting discussions, help and a nice working atmosphere.
   My present and former colleagues at Organic Chemistry and at other
       divisions and departments at KTH, who participated in creating the good
       working atmosphere.
   Lena Skowron, Ulla Jacobsson, Henry Challis and Jan Sidén for all your
       help with practical matters.
   My former mentors Fredrik Gröndahl and Mikael Lindström for many
       interesting discussions in the middle and beginning of my PhD adventure. I
       also wish to thank KTH for providing excellent mentor programs.
   My former colleagues at the House of Science for the creative and happy
       Mondays.

Sist men inte minst vill jag tacka familj, släkt och vänner för er uppmuntran
   och alla glada stunder. Världens bästa syster, Åsa, för att du alltid finns där.
   Mina föräldrar Roger och Anita Pettersson för allt stöd under åren, inte
       minst under de senaste kaotiska veckorna med intensivt skrivande och
       dagissjukor.

Till min älskade familj, Sverker, Elsa och ”snodden”.
   Sverker, utan dig hade det aldrig gått. Din kärlek betyder allt.
   Elsa, nu leker vi istället!


Appendix I

Structures of compounds mentioned in the text. First, the discussed compound classes are presented; thereafter all structures are listed in alphabetical order.

1. Terpenes

1.1 Monoterpenes

- Myrcene
- (+)-3-Carene
- Limonene
- α-Pinene
- β-Pinene

1.2 Oxygenated monoterpenes

- Citronellol
- Linalool
- Linalool
- Piperitone
- Bornyl acetate

1.3 Sesquiterpenes

- (3E,6E)-α-Farnesene
- (6E)-β-Farnesene
- (E)-α-Bisabolene
- (1R,9S)-(−)-β-Caryophyllene
- α-Longipinene
2. Green leaf volatiles

(3Z)-Hexen-1-ol  \( \text{OH} \)  (3Z)-Hexenal

3. Phenols

Methyl salicylate  Benzoic acid

3.1 Flavonoid, stilbene and lignin precursors

Phenylalanine  Cinnamoyl-CoA  \( p \)-Coumaroyl-CoA  Caffeoyl-CoA

3.2 Flavonoids

General structure of catechins

General structure of epicatechins

(2R,3S)-(+) -Catechin  (2R,3R)(-) -Epicatechin
3.3 Stilbenes

- Leucoanthocyanidin
- Proanthocyanidin of catechin and epicatechin (B-type)

- Stilbene
- (E)-Pinosylvin
- (E)-Piceid
- (E)-Resveratrol
- (E)-Astringin
- (E)-Piceatannol
- (E)-Isorhapontin
- (E)-Isorhapontigenin
Piceaside A (7''R, 8''R) and Piceaside B (7''S, 8''S)

Piceaside C (7''R, 8''R) and Piceaside D (7''S, 8''S)

Piceaside E (7''R, 8''R) and Piceaside F (7''S, 8''S)

Piceaside G (7''R, 8''R) and Piceaside H (7''S, 8''S)
4. Alphabetical order

Acetyl-CoA

(E)-Astringin

Benzoic acid

(E)-α-Bisabolene

Bornyl acetate

Caffeoyl-CoA

(1S,6R)-(+)-3-Carene

(1R,9S)-(-)-β-Caryophyllene

(2R,3S)-(+)-Catechin

Cinnamoyl-CoA

Citronellol

p-Coumaroyl-CoA

(2R,3R)-(-)-Epicatechin
cis-trans-Nepetalactone  cis-cis-Nepetalactone  trans-cis-Nepetalactone  trans-trans-Nepetalactone  Phenylalanine

Piceaside A (7''R, 8''R) and Piceaside B (7''S, 8''S)

Piceaside C (7''R, 8''R) and Piceaside D (7''S, 8''S)

Piceaside E (7''R, 8''R) and Piceaside F (7''S, 8''S)
Piceaside G (7^\text{R}, 8^\text{R}) and Piceaside H (7^\text{S}, 8^\text{S})

(E)-Piceatannol

(E)-Piceid

(1S,5S)-(−) (1R,5R)-(+) α-Pinene

(1S,5S)-(-)-β-Pinene

(E)-Pinosylvin

Piperitone

Proanthocyanidin of catechin and epicatechin (B-type)

(E)-Resveratrol
Appendix II

Denotations of terpenes and phenols in chapter 5.

**Table A1.** Denotations of terpenes in PCA’s. Compound names marked with a star are tentative identifications based on comparison with spectra in the NIST-library. The terpene class is written out for unidentified compounds, MT: monoterpene, ST: sesquiterpene, STO: oxygenated sesquiterpene and DT: diterpenoid. The m/z of the molecular ion is included within parenthesis for the diterpenoids.

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<th>T-No</th>
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Table A2. Denotations of phenols in PCA’s. uk: unknown. Only phenols for which [M-H] have been identified are included in the table, the other compounds are unknown. Compound names marked with a star are only tentative assignments and have not been confirmed by MS/MS-spectra or comparison with reference compounds.

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Description of my contribution to paper I-IV as requested by KTH.

I. I participated in the planning of the experiments. Performed the experimental work except the nepetalactone analyses on MS-instruments other than the Finnigan SSQ 7000. Did the statistical analysis and wrote the manuscript.

II. I participated in the planning of experiments. Performed the GC-MS analyses and wrote the major part of the chemistry sections in the paper.

III. I planned the experiments, performed all experimental work and wrote the manuscript.

IV. I took major part in planning of the study, prepared samples for chemical characterisation, coordinated chemical analyses and sampling, performed LC-MS and statistical analyses, and wrote the chemistry sections in the manuscript.