VIRPI VIRJAMO

Piperidine alkaloids of Norway spruce (Picea abies L. Karsten)

Relations with genotypes, season, environment and phenolics

UNIVERSITY OF
EASTERN FINLAND

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Academic Dissertation
To be presented by permission of the Faculty of Science and Forestry for public examination in the Auditorium N100 in Natura Building at the University of Eastern Finland, Joensuu, on December, 13, 2013, at 12 o'clock noon.

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ABSTRACT

In this thesis, the aim is to clarify the appearance and the role of the poorly known piperidine alkaloids of Norway spruce (*Picea abies* L. Karsten). Piperidine alkaloids are minor secondary components for Pinaceae species and assumed to be part of its defensive chemistry. Various plants parts including buds, current and previous years needles, twigs and bark were investigated. Young seedlings, as well as young and mature trees were used as study material. The thesis included four experiments: the effect of regeneration method, the effect of climatic factors, the changes during shoot development and the effect of genetic background on alkaloid composition. To get more holistic picture, also some phenolics were investigated. The main components of *P. abies* alkaloids in samples investigated during this study were epidihydropinidine, *cis*-pinidinol and 2-methyl-6-propyl-1,6-piperideine, which were detected in relatively constant concentrations (0.03, 0.01 and 0.01% dw, respectively) regardless of plant age or the plant part studied. In addition, a wide range of other piperidines, including mainly intermediates of biosynthesis or the derivatives of main components were detected. The accumulation of piperidine alkaloids in vegetative shoots occurs simultaneously in twigs and needles and was found to be closely related to bud opening. In addition, both genetic and environmental factors affected total alkaloid concentrations. Based on the studies conducted as part of this doctoral thesis, temperature is by far the most important regulatory factor for piperidine alkaloid accumulation in *P. abies*. Although the constant concentration of the major components suggest its importance in tree defence, field voles showed no avoidance of, but rather a preference for high alkaloid containing seedlings, indicating that compounds might act also as elicitors. The concentration of total alkaloids showed negative correlation with the concentration of total low molecular weight phenolics, possible referring trade-off in secondary chemistry biosynthesis. Piperidine alkaloid compounds with high potential activity and

wide occurrence in Finland could also provide added value for forestry in the search for new bioactive compounds.

Acknowledgements

"Hiiri mittaa maailmaa männynneulasella,
heinänkorrella punnitsee,
kovin miettii, mittailee,
järkeänsä käyttää:
Isolta maailma näyttää!"
Hannele Huovi (Vauvan vaaka, 1995)

LIST OF ABBREVIATIONS

C/N	carbon to nitrogen ratio
de novo	newly biosynthesized
dw	dry weight
EI	electron ionization
GC-MS	gas chromatography-mass spectrometry
HPLC	high pressure liquid chromatography
m/z	mass to charge ratio
N	sample size
p	probability of obtaining a test statistic
r_s	Spearman's correlation
Rt	retention time
SPP	solid phase partitioning
T	temperature
UV	ultraviolet
UVA	ultraviolet-A (400-315 nm)
UVB	ultraviolet-B (315-280 nm)

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on data presented in the following articles, referred to by the Roman numerals I-IV.

I   Virjamo V, Julkunen-Tiitto R, Henttonen H, Hiltunen E, Karjalainen R, Korhonen J and Huitu O. Differences in vole preference, secondary chemistry and nutrient levels between naturally regenerated and planted Norway spruce seedlings. *Journal of Chemical Ecology, 39: 1322-1334, 2013.*

II Virjamo V, Sutinen S and Julkunen-Tiitto R. Combined effect of elevated UVB, elevated temperature and fertilization on growth, needle structure and phytochemistry of young Norway spruce (*Picea abies*) seedlings. *Global Change Biology, doi: 10.1111/gcb.12464, in press.*

III Virjamo V and Julkunen-Tiitto R. Shoot development of Norway spruce (*Picea abies*) involves changes in volatile alkaloids and condensed tannins. *Resubmitted.*

IV Virjamo V and Julkunen-Tiitto R. Variation in piperidine alkaloid chemistry of Norway spruce (*Picea abies* L. Karsten) foliage in trees of diverse geographic origin grown at the same site. *Manuscript.*

AUTHOR'S CONTRIBUTION

In paper I, Virpi Virjamo (V. V.) was responsible for the alkaloid analyses, for the processing of secondary chemistry data, and was also the main author. In paper II, V. V. contributed to the founding and maintenance of the experimental field, was responsible for growth and biomass measurements, sampling and alkaloid analysis, data processing and writing the paper. In papers III and IV, V. V. planned the experiments with her main supervisor, participated in sampling, conducted data analyses and is the main author of the papers.

Contents

1 Introduction

1.1 CONIFEROUS SECONDARY COMPOUNDS AND THEIR BIOLOGICAL ROLE

In addition to primary metabolites involved functions such as respiration or photosynthesis, plants produce wide range of compounds named secondary metabolites. Many bioactive roles are put forward for secondary compounds: a defensive role against abiotic and biotic stressors, a function as an elicitor of predators, and interaction in plant-plant competition (e.g. Dudareva et al., 2004; Paschold et al., 2006; Hartmann, 2007; Li et al., 2010). The major secondary compound groups in conifers are terpenoids and phenolics. However, in addition to the well studied phenolics and terpenoids, the secondary chemistry of conifers includes volatile piperidine alkaloids.

Terpenoids, including monoterpenes, sesquiterpenes and diterpenes, are a wide group of volatile or non-volatile compounds built up of multiple isoprene units (Figure 1). In Norway spruce (*Picea abies* (L.) Karsten), terpenoids are a major ingredients of oleoresin, for which defensive properties against the European spruce bark beetle (*Ips typographus*) have been presented (Zhao et al., 2011; Schiebe et al., 2012).

Phenolics are a class of secondary compounds with an aromatic ring and one or more hydroxyl substituents. The phenolic group includes both high molecular weight compounds, such as condensed tannins, and low molecular weight compounds such as flavonoids, lignans, stilbenes and acetophenones (Figure 1). For phenolics detected in *P. abies*, a wide range of biological roles have been suggested, including UVB protection (flavonoids), defence against mammal herbivores (condensed tannins) and cold acclimation (stilbenes) (Fischbach et al., 1999; Rummukainen et al., 2007; Heiska et al., 2008). Moreover, high total phenolic concentration is regarded

as one of the major reasons for the low palatability of *P. abies* compared to that of *Pinus sylvestris* (L.) (Stolter et al., 2009).
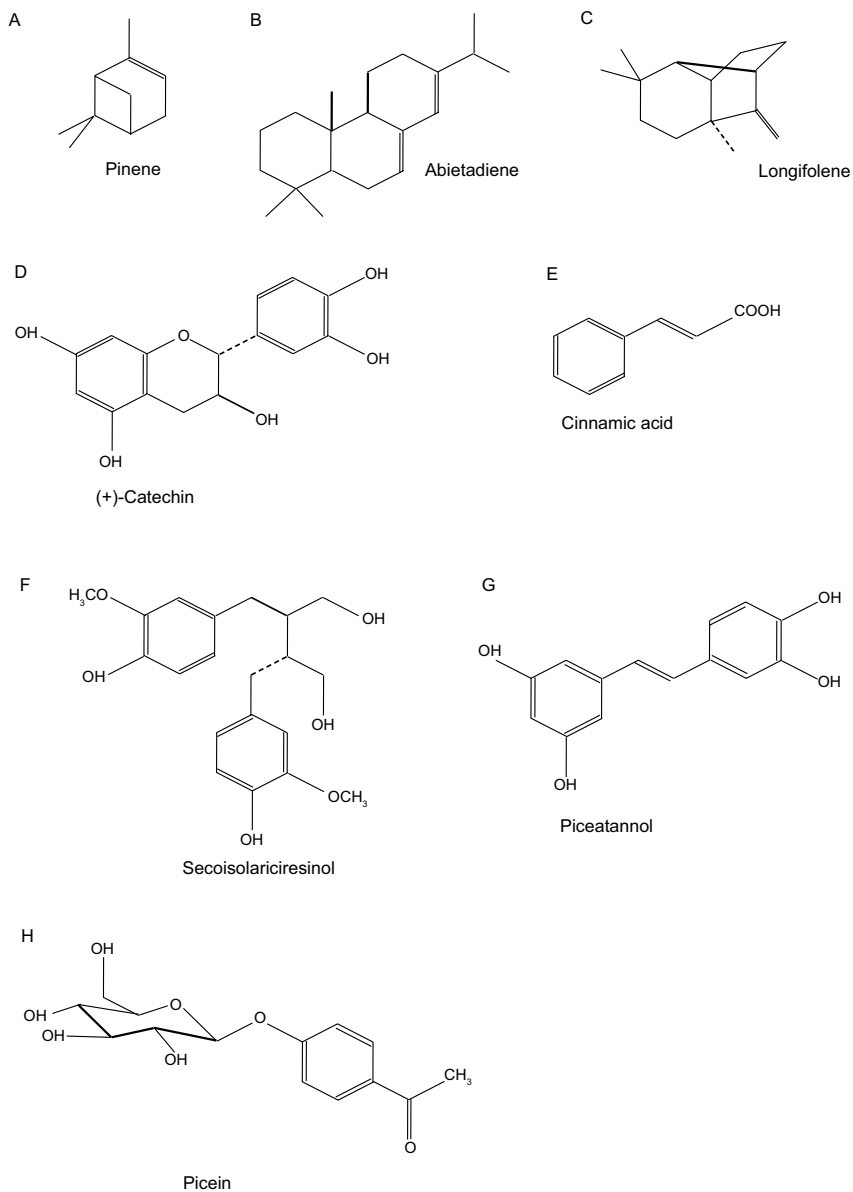
Figure 1. Examples of structures of terpenes [A) monoterpenes, B) diterpenes and C) sesquiterpenes] and phenolics [D) flavonoids, E) phenolic acids, F) lignans, G) stilbenes, H) acetophenones].

1.2 PIPERIDINE ALKALOIDS

Piperidines have relative simple structures and are defined by their six-membered heterocyclic amine ring (Figure 2). Group is named after *Piper* genus, from which wide range of piperidine compounds have been isolated (e.g. Parmar et al., 1997). In general piperidine alkaloids are biosynthesized from lysine and compounds are known for high toxicity (Seigler, 1998; Green et al., 2012). However, despite of similarities in structure, coniferous piperidine alkaloids are polyketide-derivated (Leete & Juneau, 1969; Leete et al., 1975).
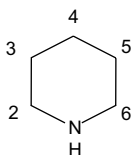
Figure 2. *Piperidine.*

The first piperidine alkaloids isolated from conifers were α-pipecoline and *cis*-pinidine, identified from *Pinus sabiniana* (Douglas) (Tallent et al., 1955). This was followed by the identification of *cis*-pinidinol and epidihydropinidine from *Picea engelmannii* (Parry ex Engelm.), leading to a still growing number of compounds (Schneider & Stermitz, 1990; Schneider et al., 1991; Tawara et al., 1993, 1999; Todd et al., 1995). Surveys conducted with various *Pinus* and *Picea* species have shown that volatile piperidine alkaloids are not related to specific species but are commonly observed in the Pinaceae family (Stermitz et al., 1994; Gerson & Kelsey 2004). Piperidine alkaloids are also encountered in the *Abies* species, but are not as widespread as those found in the *Pinus* and *Picea* species (Stermitz et al., 2000). Typically, the total alkaloid content in conifers varies from 0.03% to 0.08% of fresh weight (Tawara et al., 1993).

The numerous piperidine alkaloid compounds found in conifers are mostly 2,6-disubstituted, although monosubstituted and 4-hydroxylated compounds have also been found (Figure 3) (Tawara et al., 1993, 1999; Stermitz et al., 1994; Schneider et al.,

1995). However, 2,6-disubstituted piperidines are not only typical for conifers. Identical compounds have also been found in insect and parasite species. For example, euphococcinine was originally isolated from a beetle (*Euphorbia atoto*), *cis*-pinidinol was first identified from root hemiparasite (*Pedicularis bracteosa*), and pinidinone was first isolated from the ladybird (*Cryptolaemus montrouzieri*) (Hart et al., 1967; Brown & Moore, 1982; Schneider & Stermitz, 1990). While alkaloids detected in *Pedicularis bracteosa* are taken up from the host plant, beetles are assumed to biosynthesize piperidine alkaloids *de novo*, suggesting converged evolution (Hart et al., 1967; Brown & Moore, 1982; Schneider & Stermitz, 1990; Tawara et al., 1993).

Despite their similarities, *Pinus* and *Picea* species also show several different features in their alkaloid chemistry. In *Pinus* species either *cis*-pinidine or euphococcinine is the major compound, and virtually all piperidines are in *cis*-form (Gerson and Kelsey, 2004; Gerson et al., 2009). In the *Picea* species both *cis*- and *trans*-forms of 2,6-disubstituted piperidines are found, and epidihydropinidine is the most abundant compound along with *cis*-pinidinol (Schneider et al., 1991; Tawara et al., 1993; Stermitz et al., 1994). However, the endemic *Picea breweriana* (S. Watson) species shows an exceptional alkaloid profile having monosubstituted piperidines instead of *cis*-pinidinol and epidihydropinidine (Schneider et al., 1995). The wide range of other compounds detected in the *Picea* and *Pinus* species, in addition to the major compounds, are considered to be intermediates of biosynthesis or simple modifications of the main products (Tawara et al., 1993, 1995).

Cis-2,6-disubstituted piperidines

euphococcinine *

N-methyl-granatanone

pinidinone *

1,2-dehydropinidinone

1,2-dehydropinidinol *

cis-pinidinol *

cis-pinidine *

Trans-2,6-disubstituted piperidines

isomer of
2-methyl-6-propyl-1,6-piperideine

trans-pinidinol *

epipinidinone

2-methyl-6-propyl-1,6-piperideine

epidihydropinidine *

trans-pinidine

Monosubstituted piperidines

α-pipecoline   N-methylsedridine   N-methylpelletierine
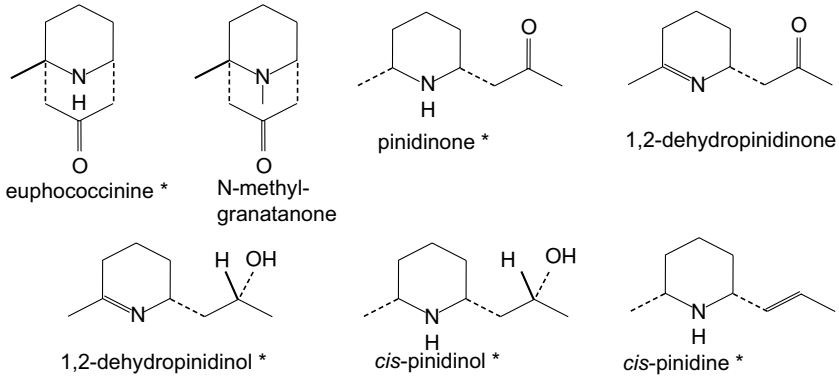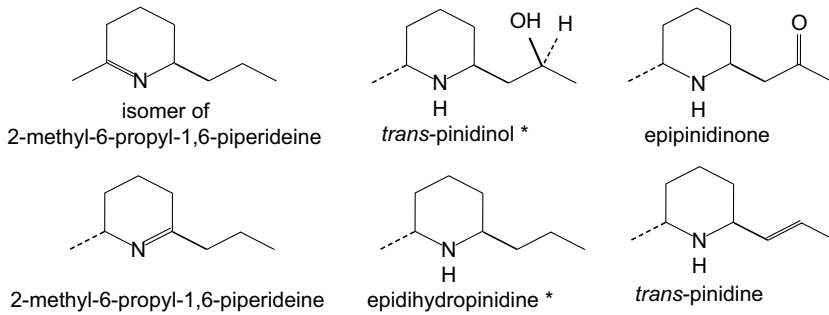
4-hydroxylated piperidines

4-hydroxy-cis-2-methyl-6-
(2-oxopropyl)piperidine

Other alkaloid compounds

Hygroline   Hygrine

4-hydroxy-cis-2-methyl-6-
propylpiperidine
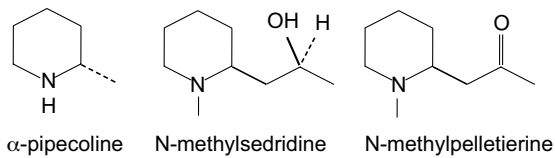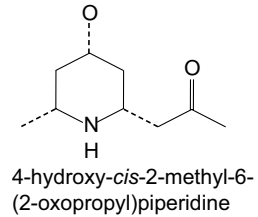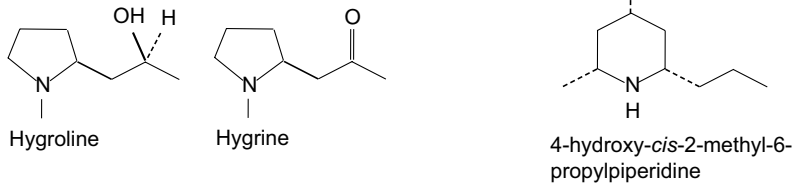
Figure 3. Structures of alkaloid compounds identified from the Pinaceae species. Compounds detected from P. abies are marked with asterisks.

1.2.1 Biosynthesis of coniferous piperidine alkaloids

Unlike many other piperidine alkaloids, the skeleton of ring structure in *cis*-pinidine is derived from acetate units and not from amino acid (Leete & Juneau, 1969; Leete et al., 1975). The precursor for the final steps of biosynthesis of *cis*-substituted piperidines is 1,2-dehydropinidinone, from which both euphococcinine and *cis*-pinidine are synthesized (Tawara et al., 1995). Synthesis of *cis*-pinidine occurs through *cis*-pinidinol and pinidinone or 1,2-dehydropinidinol (Tawara et al., 1995). In the *Pinus* genus it is typical that only one major component is synthesized actively, and some species, including *P. sylvestris*, lack the capacity to synthesize both of the end products (Stermitz et al., 1994; Gerson & Kelsey, 2004).

Contrary to the biosynthesis of *cis*-2,6-piperidines, biosynthesis of the *trans*-2,6-piperidines common in *Picea* species has not been completely explained. It is suggested that the pathway to *trans*-pinidine closely resembles that of *cis*-pinidine, involving epipinidinone and *trans*-pinidinol as intermediate compounds (Todd et al., 1995). However, synthesis of *trans*-substituted epidihydropinidine, preferably through 2-methyl-6-propyl-1,6-piperideine, might not be directly linked to synthesis of *trans*-pinidine (Todd et al., 1995).

Piperidine alkaloids accumulate early in the growth of seedlings (Tawara et al., 1995; Todd et al., 1995). Already in 6 days old *Pinus ponderosa* (Douglas ex C. Lawson) and in 9 days old *Picea pungens* (Engelm.), the seedlings showed detectable amounts of piperidine alkaloids, and concentrations rose quickly to the levels found in mature tissues (Tawara et al., 1995; Todd et al., 1995). In addition, relatively high concentrations of alkaloids have been reported to occur in new needle bundles of mature trees (Todd et al., 1995). Seasonal variation in needle alkaloid chemistry has been reported in *P. ponderosa* foliage, where the highest concentrations of piperidine alkaloids are detected in mature needles in April (Gerson & Kelsey, 1998). Similarly, current-year needles show higher alkaloid

concentrations in August than in December (Gerson & Kelsey, 1998).

1.2.2 Effect of environmental and genetic factors on coniferous piperidine alkaloids

Notable site-dependent variation in concentrations of piperidine alkaloids has been observed in *Pinus ponderosa* (Gerson & Kelsey, 1998). Thus, it is obvious that genetic and/or environmental factors affect alkaloid biosynthesis. So far, nitrogen availability has been shown to be an important factor in determining the piperidine alkaloid concentrations of *P. ponderosa* (Gerson & Kelsey, 1999a). In fact, it has been suggested that the total absence of alkaloids in some *P. ponderosa* populations detected by Gerson & Kelsey (1998) could also be a symptom of severe nutrient deficiency rather than of genetically-based biosynthesis inability (Gerson & Kelsey, 1999a).

Attempts have been made to resolve whether coniferous piperidine alkaloids play a role in the constitutive or inducible defences (Schiebe et al., 2012). However, mild herbivore pressure does not induce alkaloid production in the previous-year foliage of *P. ponderosa* (Gerson & Kelsey, 1998). In *P. abies* alkaloid levels decreased as a response to methyl jasmonate treatment, while in some of the individual trees the increase in alkaloid levels was huge (Schiebe et al., 2012). This could suggest that there are genotype-specific responses in the alkaloid biosynthesis of conifers.

Genetic control of the biosynthesis of piperidine alkaloids has recently been proven for *P. ponderosa* in a common garden (provenance) study, where seedlings of various origins were grown at the same site, in the same environmental conditions (Gerson et al., 2009). Alkaloid concentrations were also found to correlate with the parental temperature range, suggesting that temperature might be an important regulatory factor for alkaloid biosynthesis (Gerson et al., 2009).

1.2.3 Biological role of coniferous piperidine alkaloids

Alkaloids are suggested to be involved in plant-herbivore interactions (Bennet & Wallsgrove, 1994). Some compounds that are structurally similar to coniferous piperidine alkaloids, such as solenopsin and coniine, are known to be highly toxic (Jones et al., 1990; Green et al., 2012). However, the biological role of coniferous piperidine alkaloids has mostly been studied in small scale, and therefore most of the results are preliminary. High variation between populations and lack of knowledge of which individual compounds and in which concentrations they are biologically active, further complicate investigation of the ecological importance of these compounds.

Most of the study has focused on insect defence. The Mexican bean beetle (*Epilachna varivestis*) produces euphococcinine, which works as an active deterrent against ants (*Monomorium pharaonis*) and spiders (*Phidippus regius*) in laboratory tests (Eisner et al., 1986). In addition, Schneider et al. (1991) presented preliminary results suggesting moderate to high antifeedant activity against eastern spruce budworm (*Choristoneura fumiferana*) for a crude alkaloid mixture isolated from *Picea engelmannii* containing *cis*-pinidinol and epidihydropinidine. Also, a mixture of *P. engelmannii* alkaloids in an artificial diet has been shown to reduce the growth of variegated cutworm (*Peridroma saucia*) (Stermitz et al., 1994). More recently, a non-volative form of dihydropinidine has been found to have high antifeedant properties against the large pine weevil (*Hylobius abietus*) (Shtykova et al., 2008). However, total piperidine alkaloid concentrations or concentrations of major alkaloid compounds of *Picea sitchensis* (Bong.) Carrière did not correlate with white pine weevil (*Pissodes strobi*) damage in a field experiment (Gerson & Kelsey, 2002). Similarly alkaloid concentrations of *P. abies* did not show any correlation with European spruce bark beetle (*Ips typographus*) damage (Schiebe et al., 2012).

In addition to studies conducted with various insect species, the embryo toxicity of a piperidine alkaloid isolate has also been

studied (Tawara et al., 1993). A mixture of HCl-salts of piperidine alkaloids isolated from *P. ponderosa* showed high toxicity in a frog embryo test, and 100% of survivors showed malfunctions (Tawara et al., 1993). A later experiment proved that the active compound in the mixture of piperidines was *cis*-pinidine (Stermitz et al., 1994). In addition, *cis*-pinidinol and euphococcinine have been tested for antimicrobial activity (Tawara et al., 1993). While *cis*-pinidinol did not show any antimicrobial function, euphococcinine showed weak activity against gram-negative bacteria (Tawara et al., 1993). To date there have been no studies of the role of coniferous piperidine alkaloids in mammal herbivore defence.

1.2.4 *Picea abies* (L.) Karsten alkaloids

Norway spruce (*Picea abies* (L.) Karsten) is a widespread species that has a major economical and ecological role in Northern Europe, also in Finland (Skrøppa, 2003; Ylitalo, 2012). Although most surveys of coniferous piperidine alkaloids have focused on North American species, Hultin & Torssell already listed *P. abies* as alkaloid-containing plants in 1965. Tawara et al. (1993) and Stermitz et al. (1994) included *P. abies* in their studies, revealing that in mature *P. abies* tissues epidihydropinidine and *cis*-pinidinol are the major piperidine alkaloid compounds, in addition to which *trans*-pinidinol, *cis*-pinidine, euphococcinine, pinidinone and 1,2-dehydropinidinol were detected (Figure 3). Piperidine alkaloids have been detected in young and mature bark, cones, needles, roots, twig and wood tissues and even in resin of *P. abies* (Stermitz et al., 1994). More recently, Schiebe et al. (2012) conducted a study where *P. abies* alkaloids were quantitatively investigated for the first time. However, in the study by Schiebe et al. (2012) alkaloid concentrations were low, and most of the compounds, including e.g. pipecoline, not earlier detected in *P. abies*, remain tentatively identified.

1.3 AIMS OF THE THESIS

The main focus of this doctoral work was to produce fundamental knowledge on the piperidine alkaloids Norway spruce (*Picea abies*), so as to enable further, more focused studies. The ecological and economical importance of *P. abies* trees in Northern Europe justifies this study in two different ways. First, better knowledge of defensive chemistry is important for silviculture. Knowledge about environmental and genetic factors behind coniferous alkaloid chemistry can open up possibilities for modifications in nursery conditions or for tree breeding. Secondly, alongside traditional forestry, other ways of exploiting the woodlands are actively being sought. One promising possibility having a great potential is that offered by bioactive compounds. Although piperidine alkaloids of conifers are found in minor concentrations, they are assumed to be biologically highly reactive, and the available biomass for possible commercial use is significant. In order to gather a wide range of new knowledge about piperidine alkaloids of *P. abies* in relation to certain phenolics, the following topics were studied:

1. Qualitative variation of piperidine alkaloids according to tissue, age of plant and origin (I-IV).

2. Effect of regeneration method on quantitative variation of piperidine alkaloids and phenolics and its relation to field vole feeding (I)

3. Effect of fertilization, elevated temperature and UVB radiation on quantitative variation of piperidine alkaloid chemistry of *P. abies* in relation to phenolics and growth (II).

4. Seasonal variation of piperidine alkaloids and condensed tannins of *P. abies* in mature and current-year needles and twigs (I, III).

5. Effect of genetic factors on quantitative variation of piperidine alkaloid chemistry of *P. abies* (IV).

In addition, methodological aspects, biosynthesis of *trans*-2,6-substituted piperidines and bioactivity of piperidine alkaloids are discussed.

2 Material and methods

2.1 EXPERIMENTS

2.1.1 Effect of regeneration method (I)

The experimental field was located in Suonenjoki, eastern Finland. Two-year-old nursery grown seedlings were planted next to naturally regenerated *P. abies* seedlings in May 2008 (for timetable of procedures see Table 1). In September 2008, 2-year-old nursery-grown seedlings treated for autumn planting were added to the site. In November 2008, naturally regenerated seedlings, spring-planted seedlings and autumn-planted seedlings were transplanted from the field site to vole enclosures and two mature field voles (*Microtus agrestis*) were introduced for the winter. Vole damage to the seedlings was assessed after snowmelt in 2009. Seedlings were sampled for secondary chemistry analyses in January 2009 (natural and spring-planted seedlings) and September 2008 (autumn-planted seedlings) and compared in order to establish the effect of the regeneration method and any possible correlation with vole feeding preference. Moreover, naturally regenerated seedlings were sampled for secondary chemistry analyses in May 2008, November 2008 and January 2009 in order to follow seasonal changes in needles and bark. In addition to piperidine alkaloids, phenolics (Table 2) and nutrients (Finnish Forest Research Institute's Central Laboratory, Vantaa, Finland) were investigated so as to gain a more holistic picture.

Table 1. Schedule of procedures conducted and seedlings sampled for chemistry analyses (N= nutrients, SC= secondary chemistry) during experiment I. Number of individuals of each procedure is in parenthesis.

Seedling group	May 2008	September 2008	November 2008	January 2009	May 2009
Natural seedlings	Seedlings selected (240), N (8), SC (5)		Translocation to vole enclosures (60), N (8), SC (5)	N (8), SC (5)	Vole damage assessment
Spring-planted seedlings	Seedlings planted (240), N (8), SC (5)		Translocation to vole enclosures (60), N (8)	N (8), SC (5)	Vole damage assessment
Autumn-planted seedlings		Seedlings planted (120), N (8), SC (5)	Translocation to vole enclosures (60), N (8)		Vole damage assessment

Table 2. Summary of experiments conducted.

	Plant age (years)	Plant parts studied	Chemical analyses (Tot. sample number)
I	2	Previous years needles and bark	Piperidine alkaloids (60) HPLC-phenolics (60) Condensed tannins (60)
II	1	Current year needles (and bark) [a]	Piperidine alkaloids (72) HPLC-phenolics (144) Condensed tannins (144)
III	15	Buds, current year twigs[b] and needles	Piperidine alkaloids (72) Condensed tannins (72)
IV	35	Current year needles	Piperidine alkaloids (93)

[a] *Piperidine alkaloid analyses were done only in needles, phenolic compounds were also analyzed from bark*
[b] *Twigs include bark, phloem, and wood*

2.1.2 UVB, elevated temperature and fertilization (II)

The outdoor experiment was conducted in summer 2009 in Joensuu, eastern Finland with 1-year-old *P. abies* seedlings. Briefly, six replicates of six treatment and treatment combinations (Control, UVA, UVB, Temperature, UVA+T and UVB+T) were used, giving a total of 36 experimental plots (Figure 4, for more details see Nybakken et al., 2012). UV lamps

were modulated to correspond to a 32% increase compared to ambient UVB radiation, and infrared heaters were set to correspond to 2 °C above ambient temperature. At the southeast end of the each experimental plot, 8 replicates of the *P. abies* seedlings were placed in two rows, giving a total number of 288 seedlings. Half of the seedlings in each plot were randomly selected for fertilization treatment. In addition to piperidine alkaloids, phenolics (Table 2), growth and needle cross-sections (according to Kivimäenpää et al., 2001) were investigated in order to form an interactive picture of alkaloids, growth, and phenolics.
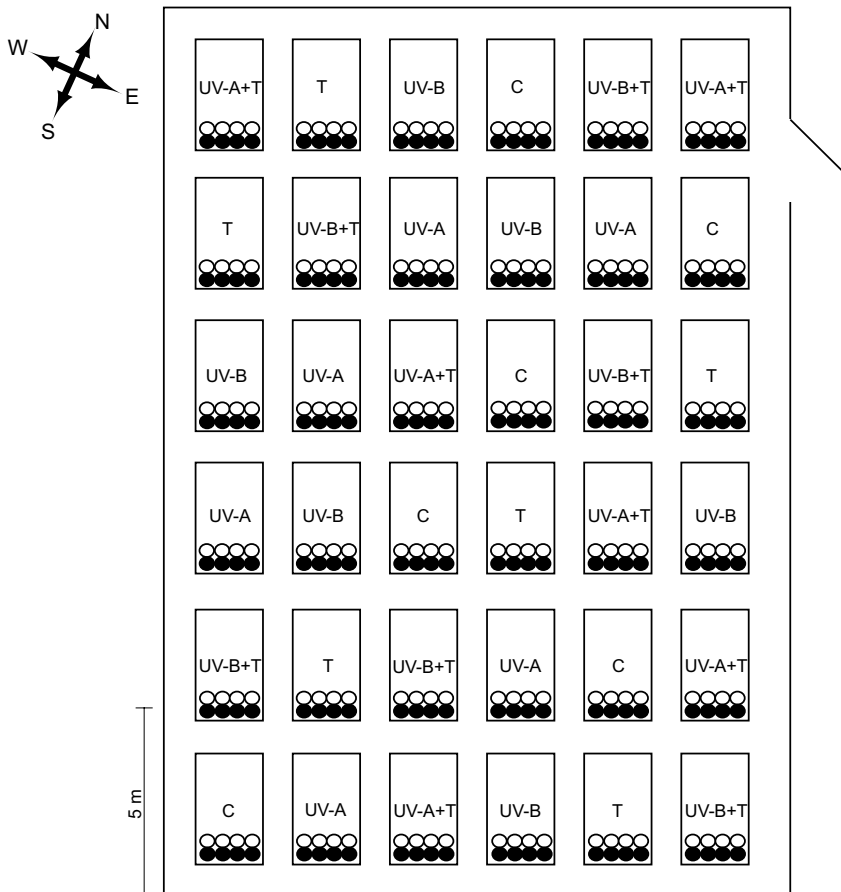
Figure 4. *Schematic representation of the UV-temperature field experiment. Open circles represent not-fertilized and closed circles fertilized P. abies seedlings.*

2.1.3 Bud opening and shoot development (III)

Bud or young shoot samples were collected six times from 1st April (dormant buds) to 16th August (mature branches) in 2012. Mixed-genotype *P. abies* trees from Kaavi, eastern Finland, were used as material. These trees were planted in 1997 to establish a commercial forest. Eight randomly selected individuals were sampled at each time point, and in addition to piperidine alkaloids, condensed tannins were also analyzed (Table 2).

2.1.4 Genetic variation between origins (IV)

Trees from twelve origins, taken from the Mikola experimental series (see Beuker et al., 1994) established in 1978 in Punkaharju, eastern Finland, were selected as study material. The origins selected for analyses cover most of the natural distribution of *P. abies* (Table 3, Skrøppa, 2003). Eight individuals of each origin (except only 5 individuals for Russia, Archangel) were randomly selected for analyses. To minimize the effect of age of needles, only current-year foliage was used for the piperidine alkaloid analyses (Table 2).

Table 3. Origins of trees planted in Punkaharju (N61°44′, E29°19′) provenance experiment according to Beuker et al. (1994).

Origin	Latitude	Longitude	Altitude (m)
Finland			
Kittilä	68°00′	24°13′	300
Kolari	67°15′	23°45′	200
Puolanka	64°44′	28°05′	250
Lieksa	63°05′	29°50′	350
Urjala	61°08′	23°28′	100
Tenhola	60°02′	23°13′	10
Russia			
Archangel	61°50′	40°00′	200
Latvia			
Kalsnava	56°43′	25°55′	95
Poland			
Augustow	53°54′	23°00′	120
Slovakia			
Cierny Vah	48°54′	20°44′	800
France			
Voges	48°04′	6°53′	800
Romania			
Vatra Dornei	47°20′	25°21′	350

2.2 SECONDARY CHEMISTRY ANALYSES

2.2.1 Alkaloid analyses

Alkaloid analyses were conducted using the solid-phase partitioning (SPP) method adapted from Wink et al. (1995) and Gerson & Kelsey (1999b). This method has not previously been used for analyses of *P. abies* alkaloids, and its suitability was thoroughly tested prior to other investigations (I). The use of

solvents and incubation time were optimized to maximize the recovery of alkaloids by the extraction procedure. The actual effectiveness and recovery of the method was tested with synthesized (±)-epidihydropinidine (I).

The sample size used was ca. 0.2–0.7g. Needles, bark, buds or twigs were homogenized in liquid nitrogen. Alkaloids were extracted from the plant material by repeated incubations in hydrochloric acid. The combined filtrate was then adjusted to basic using sodium hydroxide and a loaded Extrelut® column. The alkaloid fraction was collected from the columns with dichloromethane. The eluate was then concentrated to smaller volume for gas chromatography analyses. Identification and quantification of compounds was performed using a gas chromatograph coupled with a mass selective detector (GC-MS with EI ionization).

The compounds were identified on the basis of previous data (Hart et al., 1967; Yamazaki & Kibayashi, 1989; Tawara et al., 1993; Todd et al., 1995) and the retention order of 2,6-*trans*- and 2,6-*cis*-disubstituted piperidines (Todd et al., 1995). Synthesized (±)-epidihydropinidine was used as a standard for the quantification of all detected piperidine alkaloid compounds.

2.2.2 Phenolic analyses

Low molecular weight phenolics were extracted from needles and bark (I and II, see Table 2) according to Nybakken et al. (2012). High performance liquid chromatography (HPLC) with methanol:water gradient according to Julkunen-Tiitto et al. (1996) was used for preliminary identification and quantification of the individual compounds. Commercial standards were used for quantification of individual compounds. Further identification was performed with mass spectrometry (Julkunen-Tiitto & Sorsa, 2001, II) according to commercial standards and literature values (Strack et al., 1989; Rummukainen et al., 2007).

Condensed tannins (I, II and III, see Table 2) were analyzed acid-butanol assay for proantocyanidins according to Hagerman (2002). The condensed tannins extracted from the needle and

bark of *P. abies* according to Hagerman (2002) were used as standards in quantification.

3 Results and discussion

3.1 EXTRACTION EFFICIENCY OF *P. ABIES* ALKALOIDS

The SPP method, originally adapted from Wink et al. (1995) and optimized for *cis*-piperidines by Gerson & Kelsey (1999b), was shown to be suitable for *trans*-piperidines of *P. abies* with slight modifications. With repeated incubation of plant material and additional rinsing of the column, the residue lost during the process was minimized, and we were able to collect 92% of the epidihydropinidine extracted from the plant material (I). Because piperidine alkaloids are volatile compounds and the SPP method is a multi-step process, the actual recovery with synthetic (±)-epidihydropinidine was also tested. High recovery (88%) was obtained, dispersion between corresponding samples was very small and reference response was linear in the tested concentrations (I). While the alkaloid concentrations detected in various *P. abies* tissues here are considerably higher (µg/g dw compared to ng/g dw) than those reported earlier by Schiebe et al. (2012), they are on the same scale as those detected by a comparable method in *Picea sitchensis*, *Picea omorika* (Pancic) Purk. and *Pinus* species, further supporting the reliability of the method used for analyses (e.g. Gerson & Kelsey, 1998; Stermizt et al., 2000; Gerson & Kelsey, 2004).

3.2 PIPERIDINE ALKALOIDS IN *P. ABIES*

Before my study, seven fully identified piperidine alkaloid compounds were known to occur in *P. abies* tissues (Table 4). In my samples all these compounds, except for *cis*-pinidine, were detected, and five other alkaloid compounds were recognized for the first time for *P. abies*. The newly identified compounds are 2-methyl-6-propyl-1,6-piperideine (I), *trans*-pinidine (I),

epipinidinone (III), 1,2-dehydropinidinone (III) and isomer of 2-methyl-6-propyl-1,6-piperideine (IV). Moreover, two new 1,6-imines, 1,6-dehydropinidinol and 1,6-dehydropinidinone, were tentatively identified (Figure 5, III). The number of individual piperidine alkaloid compounds in *P. abies* is likely to be even greater, since I detected four compounds with a mass peak at 98 indicating piperidine alkaloids, but they remain only partly identified (III, IV).

Table 4. *Piperidine alkaloid compounds identified from P. abies.*

Rt	Compound	EI mass spectrum, m/z (%)	Identified from *P. abies*
-	*cis*-pinidine	-	Tawara et al., 1993
3.48	2-methyl-6-propyl-1,6-piperideine	139 [M] (24), 124 (44), 111 (61), 96 (100), 70 (26), 42 (19), 41 (19)	(I)
3.52	epidihydropinidine	141 [M] (1), 126 (7), 98 (100), 81 (5)	Tawara et al., 1993
3.59	*trans*-pinidine	139 [M] (46), 124 (100), 111 (28), 96 (68), 82 (46), 55 (26), 41 (32)	(I)
3.64	isomer of 2-methyl-6-propyl-1,6-piperideine	139 [M] (69), 110 (100), 97 (91), 96 (96), 83 (15), 82 (73)	(IV)
4.32	pinidinone	155 [M] (15), 140 (31), 112 (26), 98 (100), 82 (47), 70 (10)	Tawara et al., 1993
4.61	epipinidinone	155 [M] (16), 140 (28), 112 (15), 98 (100), 82 (35), 70 (21)	(III)
4.67	*cis*-pinidinol	157 [M], 142 (12), 98 (100), 82 (9), 70 (7)	Tawara et al., 1993
4.76	tentative 1,6-dehydropinidinol	155 [M] (8), 137 (33), 122 (62), 111 (100), 96 (78), 83 (54), 68 (60), 42 (73)	(III)
4.84	1,2-dehydropinidinone	153 [M] (2), 110 (100), 96 (1), 82 (21), 71 (8)	(III)
4.95	*trans*-pinidinol	157 [M] (5), 142 (16), 98 (100), 82 (11), 70 (9)	Tawara et al., 1993
5.04	1,2-dehydropinidinol	155 [M] (10), 140 (8), 110 (13), 97 (100), 96 (54), 82 (36)	Stermitz et al., 1994
5.29	euphococcinine	153 [M] (51), 110 (100), 96 (46)	Stermitz et al., 1994
5.44	tentative 1,6-dehydropinidinone	153 [M] (23), 111 (9), 110 (17), 97 (13), 96 (100), 94 (14)	(III)

Figure 5. *Tentatively identified 1,6-imines.*

Most of the newly reported piperidine alkaloids detected in *P. abies* are minor components, and many of them have been described earlier as intermediates of an alkaloid biosynthetic pathway for other Pinaceae species (Tawara et al., 1995; Todd et al., 1995). However, it seems that, in addition to epidihydropinidine and *cis*-pinidinol, 2-methyl-6-propyl-1,6-piperideine, reported in (I), should be considered a major component for *P. abies*, based on its consistently high concentrations both in juvenile and mature tissues (Table 5). This differs from other studied *Picea* species (*P. sitchensis*, *P. omorika* and *P. pungens*), where 2-methyl-6-propyl-1,6-piperideine is a minor or non-detected component in mature needles (Todd et al., 1995; Stermitz et al., 2000; Gerson & Kelsey, 2002).

Altogether, nearly 300 individual bud, needle, bark or twig samples were analyzed for this study. Piperidine alkaloids were detected in all the investigated samples, with the exception of the two individual needle specimens. Moreover, considerable amounts of piperidine alkaloids were found in all the plant parts studied, with the exception of mature wood, where the concentrations were low, less than 15% of that detected in bark or needles. In general, twigs (and bark) were richer in total piperidine alkaloids than was foliage (I, III). This is typical for piperidine alkaloids, also in other *Picea* species (Stermitz et al., 1994, 2000). Qualitatively, mature tissues did not differ from one another (Table 5). However, there are significant differences between juvenile and mature tissues, both in quantity and in quality (III). In agreement with my results, it has been reported earlier that the concentration of piperidine alkaloids in *P. ponderosa* needles changes significantly as they mature (Gerson

& Kelsey, 1998). Some alkaloids are clearly related to juvenile tissues, for example pinidinone and 1,2-dehydropinidinone, while *trans*-pinidine was found only in mature tissues (Table 5).

Table 5. Variation in selected compounds (concentration > 50 µg/g dw) in different tissues of P. abies. The age of the trees in years is shown in parenthesis.

	Epidihydropinidine	Cis-pinidinol	2-methyl-6-propyl-1,6-piperideine	Trans-pinidinol	Trans-pinidine	Euphococcinine	1,2-dehydropinidinone	1,2-dehydropinidinol	pinidinone
Dormant buds	x	x	x					x	
Young needles	x	x	x	x		x	x	x	x
Mature needles (1)	x	x	x		x				
Mature needles (15)	x	x	x		x	x			
Mature needles (35)	x	x	x		x				
Young twigs	x	x	x				x	x	x
Mature twigs (15)	x	x	x	x				x	
Mature bark (1)	x	x	x	x	x				

During this study, I analyzed young 1–2-year-old seedlings (I, II), 15-year-old trees (III) and 35-year-old trees (IV). When the results for mature foliage are compared, it seems that piperidine alkaloid composition is not related to the age of the trees (Table 5). This could indicate that piperidine alkaloids are not related to specific, age-related defence mechanisms but rather are needed throughout the long life span of *P. abies*.

3.3 TIMING OF BIOSYNTHESIS OF PIPERIDINE ALKALOIDS

On the basis of my results, it seems that in *P. abies* the biosynthesis of piperidine alkaloids occurs mainly in newly emerged shoots, immediately after bud opening. Thus, I suggest that the temporary and significant increase of intermediate

compounds in immature plant tissues is related to the relatively narrow time window for piperidine alkaloid biosynthesis in *P. abies* (III). No similar follow-up of alkaloid levels from bud to mature shoot has so far been conducted earlier for coniferous piperidine alkaloids, but it seems that species-specific differences might exist. In *P. pungens*, new needle bundles do contain different alkaloids than mature branches, indicating that the timing of synthesis might be similar to that of *P. abies* (Todd et al., 1995). However, in *P. abies*, concentrations of 2-methyl-6-propyl-1,6-piperideine and epidihydropinidine are consistently high (Table 5, III). This contradicts earlier results obtained from *P. pungens*, where 2-methyl-6-propyl-1,6-piperidine was found only in new needle bundles and it was missing from mature needles, while the opposite was true for epidihydropinidine (Todd et al., 1995).

The effect of the season on alkaloid levels was dependent on the age of the plant tissue. The active biosynthesis of piperidines, which occurred early season in current-year tissues, was not detected from previous year tissues (I). This supports the theory that the end products of piperidine alkaloids are relatively stable as also presented earlier by Gerson & Kelsey (1998). However, contrary to my results, the total alkaloid concentrations of previous-year foliage of *P. ponderosa* have been reported to vary during the growing season and to be highest in April (Gerson & Kelsey, 1998). Thus it seems that biosynthesis of alkaloids in *Pinus* genus might be regulated differently compared to the process in the *Picea* genus, and/or species-specific differences might appear. Either way, results from individual species should not be generalized to cover all the Pinaceae family.

3.4 BIOSYNTHESIS OF *TRANS*-2,6-PIPERIDINES

While the final steps of the biosynthesis of *cis*-2,6-piperidines have been described comprehensively (Tawara et al., 1995, Figure 6), the biosynthesis of *trans*-2,6-piperidines detected

mainly in *Picea* species remains partly unsolved (Todd et al., 1995). This is especially problematic because, on the basis of empirical data, the synthesis of epidihydropinidine and *trans*-pinidine are unlikely to be closely connected (Todd et al., 1995). Simultaneously with the appearance of intermediates of *cis*-2,6-piperidines, high concentrations of tentatively identified 1,6-dehydropinidinol and 1,6-dehydropinidinone are detected (III). Basing my hypothesis on the timing of appearance and on the structural similarities to the *cis*-2,6-piperidine biosynthesis pathway, I suggest that the new, partially identified compounds detected in (III) could constitute the missing link between epidihydropinidine and *trans*-pinidine biosynthesis. 1,6-dehydropinidinone would function as a precursor for both the synthesis of *trans*-pinidine (through epipinidinone and *trans*-pinidinol) and the synthesis of epidihydropinidine (through 1,6-dehydropinidinol and 2-methyl-6-propyl-1,6-piperideine) (Figure 6). However, these compounds were not fully identified, and a labelling test would be needed to confirm the biosynthesis routes for *trans*-substituted end products.
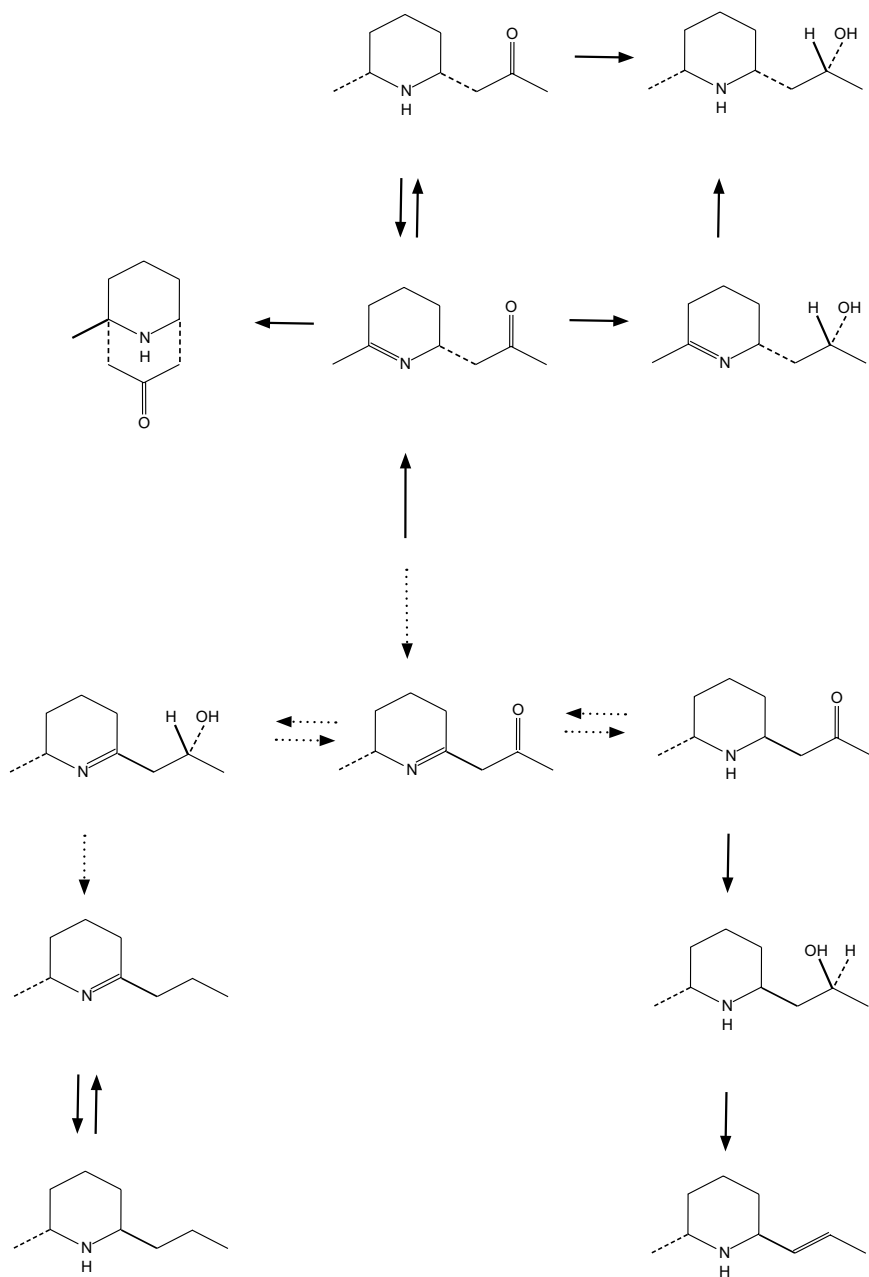
Figure 6. Biosynthesis of 2,6-cis- and trans-piperidine alkaloids of *P. abies*. Solid arrows refer to connections suggested by Tawara et al. (1995) and Todd et al. (1995) and dotted arrows refer to connections suggested on the basis of empirical data (III).

3.5 EFFECT OF ENVIRONMENTAL FACTORS

Fertilization and UVB treatment did not show any effect on the alkaloid chemistry of *P. abies*. In contrast, temperature, tree origin and regeneration method were found to affect piperidine alkaloid concentrations significantly (Table 6).

Table 6. Effects of environmental and genetic variables on concentration of piperidine alkaloids.

Factor	Piperidine alkaloid concentration
Fertilization	No effect (II)
Temperature	Elevated temperature ↑ (II)
UVB	No effect (II)
Tree origin	High latitude ↑ and low annual temperature sum ↑ (IV)
Regeneration method	Autumn-planted seedlings ↑ (I)

Naturally regenerated and spring-planted seedlings were found to have significantly less piperidine alkaloids in their bark than autumn-planted seedlings (I). Similar tendency was detected for needles (I). This could indicate the effect of ample fertilization on the autumn-planted seedlings during a long period in greenhouse conditions increasing the alkaloid concentrations. However, fertilization did not affect the needle alkaloid concentrations of young *P. abies* seedlings in the outdoor experiment (II) although fertilization has earlier shown to increase alkaloid concentrations of foliage in *P. ponderosa* (Gerson & Kelsey, 1999a). It has been suggested that the timing of fertilization could be critical for the alkaloid response of conifers (Gerson & Kelsey, 1999a). If biosynthesis of piperidine alkaloids in *P. abies* occurs immediately after bud opening (III), this would mean that the plant should be fertilized prior to or at the beginning of the active growing season in order to modulate alkaloid concentrations.

It seems that, of the range of environmental factors investigated during this doctoral study, elevated temperature is by far the most important factor affecting the piperidine alkaloid

chemistry of *P. abies*. Only a few degrees' rise in temperature resulted in significantly higher alkaloid concentrations in the outdoor experiment, mainly affecting concentrations of *cis*-pinidinol and 2-methyl-6-propyl-1,6-piperideine (II). The increase was significant even though the temperature treatment did not start before or during the time of active biosynthesis but later on in the growing season. Thus elevated temperature seems to be capable of reactivating alkaloid biosynthesis even in mature needles. Moreover, the annual temperature sum of seed origins correlated with the trees' alkaloid chemistry in the provenance experiment, which further supports the observation (IV). Similarly to our results, *P. ponderosa* piperidine alkaloid concentrations are reported to correlate with the temperature range of parental trees (Gerson et al., 2009). It seems that temperature, in addition to nitrogen supply, is the major force affecting total alkaloid concentrations in Pinaceae species. Since the effect of temperature is so obvious, it is also likely that current temperature controls the timing of alkaloid biosynthesis in newly emerging vegetative shoots (III). Similarly, the higher concentrations of piperidine alkaloids in autumn-planted seedlings compared to spring-planted or natural seedlings might be related to greenhouse conditions of elevated temperature in addition to ample fertilization (I).

3.6 EFFECT OF GENETIC FACTORS

Surveys conducted with *P. abies* before this doctoral study have been carried with material collected from two individual locations, Bavarian forest, Germany (Tawara et al., 1993; Stermitz et al., 1994) and Parismåla, Southern Sweden (Schiebe et al., 2012). In *P. ponderosa* population-dependent differences in alkaloid chemistry have been reported and the differences have been confirmed to have a genetic background (Gerson & Kelsey, 1998; Gerson et al., 2009). To clarify possible inter-species differences in *P. abies*, trees from 12 geographic origins having grown in the same place for 35 years were investigated. The

most northerly origins showed significantly higher total alkaloid concentrations than did those of southern origins (Table 6, IV). This indicates that clear genetic variation exists between origins. However, on the basis of the provenance experiment it is not possible to conclude whether these differences are the same when the trees have been grown in their original locations.

Altogether, despite significant environmental and genetic differences between the treatments and individuals investigated here, the magnitude of concentrations was relatively similar throughout the various experiments. However, the piperidine alkaloid concentrations reported from older *P. abies* trees (about 90 to 100 years old) originating from southern Sweden are substantially lower than the concentrations detected here (Schiebe et al., 2012). Individual populations where alkaloid concentration is close to zero have been earlier reported from *P. ponderosa* (Gerson & Kelsey, 1998) and it is possible that similar the scale of variation could also exist in *P. abies*. Unfortunately, no corresponding tree origins were investigated during this study. Also, some of the differences may be related to the methodology used for the extraction (simple methanol extraction instead of SPP) and quantification (pinidine and dehydropinidine standards instead of epidihydropinidine) of alkaloid compounds (Schiebe et al., 2012; I).

3.7 NEW INSIGHTS INTO THE BIOLOGICAL ROLE OF *P. ABIES* ALKALOIDS

In *P. abies*, the major components (epidihydropinidine, *cis*-pinidinol, and 2-methyl-6-propyl-1,6-piperideine) are found in relatively constant concentrations whether different plant tissues or different-aged trees are investigated (Table 5). However, nitrogen-containing compounds such as alkaloids are thought to be expensive for plants, especially in nitrogen poor boreal forests (Bryant et al., 1983), and thus constant concentrations might be regarded as an indicator of the importance of these compounds to *P. abies* defence.

The strongest evidence of antifeedant effects has been found with regard to non-volatile forms (HCL-salts) of piperidine alkaloids (Stermitz et al., 1994; Shtykova et al., 2008). However, in conifers piperidine alkaloids are present in volatile forms (Tawara et al., 1993). When the ecological role of piperidine alkaloids is studied, the ways in which they act in defence of the plants should also be considered carefully. Plants emit a wide range of volatile compounds as positive or negative signals for herbivores and their predators (e.g. Dudareva et al., 2004). However, to my knowledge, there are no studies where these alkaloids are found to be emitted in the volatile fractions of conifers. As piperidine alkaloids are water soluble (Gerson & Kelsey, 2002) and possibly toxic (Tawara et al., 1993), the plant might store these compounds in the vacuoles. The release of piperidine alkaloids could thus occur as a response to specific signal or simply as a result of wounding.

Enantiomers and stereoisomers can display antagonist biological effects (Mori, 1997). Thus, an interesting question is whether the different major components of the *Pinus* and *Picea* genus have the same effects on defence. In Northern Europe, along with *P. abies*, Scots pine (*P. sylvestris*) also still retains a role in forestry. *P. sylvestris* needles have been reported to contain only euphococcinine and 1,2-dehydropinidinol, which means that it has a very different alkaloid chemistry to that of *P. abies* (Stermitz et al., 1994). Moreover, in preliminary investigations related to this doctoral project, no alkaloids could be detected in *P. sylvestris*, suggesting that contrary to the case with to *P. abies*, scale of quantitative variation can be large.

To my knowledge, the effect of coniferous piperidine alkaloids on mammal browsing has not been studied earlier. Here, I investigated whether field vole (*Microtus agrestis*) preference for differently grown *P. abies* seedlings correlates with the seedlings' secondary chemistry (I). While significant differences in alkaloid chemistry were found in bark, the voles showed no avoidance of, but rather preference for the highest alkaloid concentrations. This could suggest that voles are tolerant to *P. abies* alkaloids, or that they even use them as

feeding cues, indicating the good nitrogen status of the seedlings. A similar double role, as an elicitor for some species and a deterrent for others, has earlier been proposed for pyrrolizide alkaloids of the *Senecio* species (Macel, 2011) as well as for many other plant compounds (e.g. salicylates: Heiska et al., 2008; Kosonen et al., 2012).

There are several major mammal pests of *P. abies* that have still not been investigated for their responses to varying alkaloid concentrations. However, on the basis of earlier data, it might be more likely that these compounds display defensive properties against insects (Eisner et al., 1986; Schneider et al., 1991; Stermitz et al., 1994; Shtykova et al., 2008). An interesting detail is the major role of 2-methyl-6-propyl-1,6-piperideine in *P. abies* alkaloid chemistry. It is found as a minor compound in *P. sitchensis,* but the highest concentrations have been reported in families resistant to white pine weevil (*Pissodes strobi*) (Gerson & Kelsey, 2002). However, more research is needed to confirm the active role of coniferous piperidine alkaloids as defensive compounds.

## 3.8 PIPERIDINE ALKALOIDS IN RELATION TO OTHER SECONDARY COMPOUNDS AND GROWTH

The secondary chemistry of *P. abies* also includes other chemically distinct groups of compounds. In this study, to achieve a more holistic picture of *P. abies* responses to the tested environmental factors, growth (II) and another group of secondary metabolites, phenolics (I-III) were also investigated. The third important group of secondary compounds in conifers, terpenoids, was not included in this study.

Growth and production of defensive compounds compete for the same limited resources (e.g. Bryant et al., 1983; Herms & Mattson, 1992). The carbon/nutrient balance hypothesis predicts that in nutrient-limited plants, carbon is allocated to carbon-based secondary chemistry (e.g. phenolics), while in nutrient-rich conditions, the plant allocates carbon to growth (Bryant et

al., 1983). Moreover, in carbon-limited conditions, resources would be allocated to the production of nitrogen-based secondary compounds (e.g. alkaloids). For *P. ponderosa*, a slight negative correlation between seedlings size and foliage alkaloid concentrations has earlier been reported (Gerson et al., 2009). Here, in experiment (II), no correlation between total biomass allocation and needle alkaloid concentration were found (r_s = -0.06, *p* = 0.065, N = 70). However, the use of concentrations in allocation comparison has been criticized, since plants do not produce concentrations but quantities (Koricheva, 1999). In experiment (IV), I used graphic vector analyses to compare organ size, concentrations and contents of piperidine alkaloids in order to overcome the possible problem of a dilution or concentration effect when comparing the concentrations (Koricheva, 1999; Veteli et al., 2007). The studied origins of *P. abies* showed either excess biosynthesis or reduced biosynthesis compared to the reference origin (IV). This can be interpreted as indicating that trade-off in southern origins (translocated to north) favours biomass allocation and in northern origins (translocated to south), allocation to alkaloid production. When changes in temperature are known to affect to both the nitrogen and the carbon dioxide assimilation of the plant, it can be hypothesized that the shift in trade-off is related to the C/N ratio.

Interestingly, I also found that concentrations of low molecular weight phenolics correlate negatively with concentrations of piperidine alkaloids in needles (Figure 7). Previous studies have shown that in tobacco, which produced both alkaloic and phenolic secondary compounds, a change in nitrogen status causes a considerable shift in secondary metabolite production (Fritz et al., 2006). Also in conifers, nutrition (i.e. nitrogen supply) is an important factor regulating both concentrations of phenolics and concentrations of piperidine alkaloids (Gerson & Kelsey, 1999a; Edenius et al., 2012). However, the apparently short time window when fertilization affects the alkaloid chemistry of *P. abies* does not support the conclusion that the correlation detected here is a

direct result of nutrient conditions. It is more likely that the observed correlation is related to the opposite responses of phenolics and piperidine alkaloids to elevated temperature (II; Riikonen et al., 2012). However, further studies would be needed to confirm this hypothesis.
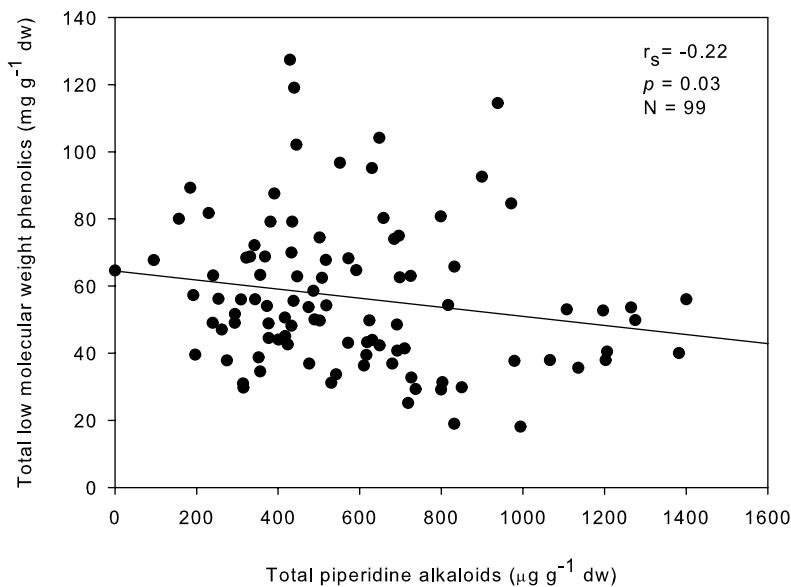
Figure 7. Correlation between low molecular weight phenolics and piperidine alkaloids (I, II) in needles of young P. abies seedlings. r_s refers to Spearman's correlation.

3.9 HUMAN USE OF *P. ABIES* ALKALOIDS

Being toxic often means having medicinal properties, and the history of alkaloid research has been the history of pharmacology (e.g. Raskin et al., 2002; Zenk & Juenger, 2007). Thus piperidine alkaloids of *P. abies* may also have uses in a commercial sense. For instance, a wide range of pharmaceutical applications, from antioxidant to anti-carcinogenic properties, have been put forward for pomegranate extract, which among other compounds contains monosubstituted piperidine

alkaloids (Teixeira da Silva et al., 2013). Although alkaloids are minor components in *P. abies*, their potential activity might be achieved with very low concentrations and thus, the high available biomass opens up various possibilities in the refining chain of *P. abies* trees.

Another possible use for bioactive compounds, such as piperidine alkaloids, would be as repellents. While many of traditionally used herbivore repellents have more recently proved to be harmful for the environment, the use of plants' own defensive compounds has taken on a higher profile (e.g. Bratt et al., 2001; Isman, 2006; Koul, 2008). In a wax coating experiment, it has already been shown that a non-volatile form of dihydropinidine is highly effective against pine weevil feeding (Shtykova et al., 2008). However, lack of knowledge about the toxicity of coniferous piperidine alkaloid compounds for other species is so far preventing its further use.

Interestingly, new shoots of *P. abies* are used as a food supplement and in herbal medicine (Piippo, 2004). Newly emerging shoots are consumed directly from the tree, as syrup and as commercial products, such as candies, drinks and cough drops. However, a similar type of traditional remedy use of pine needles in North America has been questioned because of the high teratogenic properties of *cis*-pinidine in frog embryo tests (Tawara et al., 1993). Newly emerging vegetative shoots of *P. abies* contain significantly higher total alkaloid concentrations than do mature tissues (III). However, alkaloid compounds in *P. abies* are quantitatively minor components, and it is possible that they decompose during product processing, such as the boiling of syrup from new shoots. Nevertheless, the toxic and possible teratogenic properties of *P. abies* alkaloids and their stability in products would need further investigation.

4 Conclusions

This study presents new information regarding the occurrence and variation of piperidine alkaloids in *P. abies*. Knowledge about concentrations and about qualitative composition in tree organs is essential when considering their role in tree defence or planning experimental feeding tests. Temperature is the main factor affecting the piperidine alkaloid chemistry of *P. abies* and even few degrees increase in temperature resulted significant increase in alkaloid concentrations. This is especially interesting when it is predicted that climate change will raise temperatures by 1–5 °C in Finland and in Northern Europe by the 2050s (Jylhä et al., 2004; IPCC, 2007). Based on our results, global climate change will lead to changes in *P. abies* defensive chemistry, possibly causing a shift in the palatability of *P. abies.* Genetic differences found among populations also open up possibilities for tree breeding in silviculture. However, if both elicitor and deterrent roles exist for coniferous piperidine alkaloids, unexpected herbivore damage might appear in future climate. For an economically important tree, such as *P. abies*, this may lead to losses.

Several differences were found between *P. abies* studied here and other coniferous species reported in literature. Species-specific differences underline that the alkaloid chemistry of the Pinaceae family or even the *Picea* genus should not be generalized.

Despite the new knowledge gathered in this study, further studies are needed in order to clarify the ecological role of piperidine alkaloid compounds. The toxicity of the compounds should also be tested, as newly emerged shoots of *P. abies* are used in human nutrition.

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Piperidine alkaloids
of Norway spruce
(Picea abies L. Karsten)
Relations with genotypes, season,
environment and phenolics

Secondary chemistry of economically important Norway spruce (Picea abies L. Karsten) involves poorly known piperidine alkaloids in addition to terpenoids and phenolics. This thesis provides knowledge about seasonal, environmental, and genetic variance of piperidine alkaloids, both qualitatively and quantitatively. Alkaloids compounds are assumed to be part of plants defence system and this knowledge may be useful for understanding plant-herbivore relationships and predicting how these relationships may response to changing climate.

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