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Interactions between host trees and fungi associated with the spruce bark beetle *(Ips typographus)*

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ACADEMIC DISSERTATION IN FOREST PROTECTION

To be presented, with the permission of the Faculty of Forestry of the University of Joensuu, for public criticism in Auditorium B1, of the Natura Building, Yliopistokatu 7, Joensuu, on November 1st 2002, at 12 o'clock noon.

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Abstract

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Among the European scolytids the spruce bark beetle, *Ips typographus*, is the most important species in conifers. Some of the fungi associated with spruce bark beetles have a role in overwhelming the resistance of vigorous Norway spruce, *Picea abies*. The species composition of ophiostomatoid fungi associated with *I. typographus* was investigated at different population levels of beetle in Finland and France. The fungal flora varied at different study areas. In Finland the most frequent ophiostomatoid species were *Ophiostoma penicillatum*, *O. piceaperdum* and *O. bicolor*. In France, in addition of *O. bicolor* and *O. penicillatum*, *Ceratocystis polonica* and *Ceratocystiopsis minuta* were frequent species. The frequency of highly pathogenic *C. polonica* was lower in Finland than in post-epidemic areas of spruce bark beetle in France.

Interactions between the host and the pathogen were studied after artificial inoculation of Norway spruce with *C. polonica*, the most pathogenic fungus associated with *I. typographus*. In experiment, plots of mature Norway spruce were fertilized with nitrogen, phosphorus or combination of nitrogen, phosphorus and potassium. One year after fertilization the trees were artificially infected with *C. polonica*. Changes in the main secondary compounds of Norway spruce, the stilbenes and terpenes, and soluble carbohydrate concentrations of phloem were studied in relation to the nutrient status of trees.

The response of stilbenes to fungal inoculation was qualitative. The concentration of stilbene glycosides in the phloem decreased. Corresponding stilbene aglycones were more frequent inside the reaction lesion. Fungal inoculation caused a strong quantitative response in terpenes. The total terpene concentration of the phloem increased to almost 100 times greater near the inoculation site compared to the constitutive values. N fertilization significantly reduced the total terpene and total stilbene aglycone concentrations near the inoculation sites. Thus, N fertilization may reduce the ability of Norway spruce to defend itself against fungal pathogens.

The concentration of total soluble carbohydrates in the outer border of the lesion was significantly decreased in P-fertilized trees compared to corresponding unfertilized trees. However, changes in soluble carbohydrate concentration caused by fungal inoculation were more pronounced than changes caused by fertilization. The main soluble carbohydrate was sucrose. Near the site of fungal inoculation the concentration of total soluble carbohydrates decreased significantly compared to corresponding values in unwounded phloem. N and NPK fertilization treatments increased radial growth of the stem and the vigour indices. Despite the increased radial growth of the stem, the only indication that enhanced growth might reduce the level of resistance was the modest positive correlation between lesion length and radial growth of the stem.

Key words: *Ceratocystis,* induced defense, monoterpenes, *Ophiostoma,* phenolics, *Picea abies,* soluble carbohydrates, stilbenes

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Tiivistelmä

Viiri, H. 2002. Kirjanpainajan *(Ips typographus)* seuralaissienten ja kuusen väliset vuorovaikutussuhteet. Metsäntutkimuslaitoksen tiedonantoja 864. ISBN 951-40-1846-X. ISSN 0358-4283.

Kirjanpainaja *(Ips typographus)* on kuusella esiintyvä tuhohyönteinen, joka kuljettaa mukanaan puuhun patogeenisiä värivikaa aiheuttavia sieniä. Tässä työssä tutkittiin kirjanpainajan pyydystysmenetelmien vaikutusta seuralaissienilajiston rakenteeseen (osajulkaisu I), seuralaissienilajistoa Suomessa ja Ranskassa (osajulkaisut I ja II), sekä patogeenisen seuralaissienen *(Ceratocystis polonica)* aiheuttamia muutoksia kuusen terpenoidien, stilbeenien ja liukoisten hiilihydraattien määrissä erilaisilla lannoitetasoilla (osajulkaisut III ja IV).

Sienilajisto vaihteli eri tutkimusalueilla. Suomessa kirjanpainajan endeemisellä esiintymisalueella yleisiä seuralaissieniä olivat *Ophiostoma penicillatum*, *O. piceaperdum* and *O. bicolor*. Ranskassa kirjanpainajaepidemian jälkeen *O. bicolor*, *O. penicillatum*, *Ceratocystis polonica* ja *Ceratocystiopsis minuta* olivat yleisiä seuralaissieniä. Erittäin patogeeniseksi todettu *C. polonica* oli harvinaisempi Suomessa kuin Ranskassa.

Isäntäpuun ja patogeenisen sienen välisiä vuorovaikutussuhteita tutkittiin ymppäämällä keinotekoisesti *C. polonica* sientä eläviin terveisiin kuusiin. Koepuita oli lannoitettu vuosi ennen sieni-infektiota typellä, fosforilla tai NPK-lannoitteella. Rungon sekundääriaineista stilbeenit reagoivat sieniymppäykseen kvalitatiivisesti, mutta terpeenit kvantitatiivisesti. Terpeenien määrä lisääntyi yli 100-kertaiseksi lähellä sieni-infektion kohtaa vioittamattomiin puihin verrattuna. Stilbeenien glykosidien määrä aleni lähellä sienen ymppäyskohtaa merkittävästi. Lisäksi stilbeenien aglykoneita tavattiin merkittävästi useammin lähellä sienen ymppäyskohtaa kuin vioittamattomassa nilassa. Typpilannoitus alensi kokonaisterpeenien ja stilbeenien aglykonien kokonaismäärää infektiokohdan läheisyydessä, mikä osoittaa, että typpilannoitus voi heikentää kuusen kestävyyttä tuhonaiheuttajia vastaan.

Sieniymppäyksen aiheuttamat muutokset liukoisissa hiilihydraateissa olivat merkittävimpiä kuin lannoituksen aiheuttamat muutokset. Lähellä sienen ymppäyskohtaa liukoisten hiilihydraattien kokonaismäärä aleni merkittävästi verrattuna vioittamattomien puiden nilaan. Yleisin liukoinen hiilihydraatti oli sakkaroosi. Typpi- ja NPK-lannoitukset lisäsivät rungon paksuuskasvua ja puiden elinvoimaisuusindeksiä.

Avainsanat: *Ceratocystis,* fenoliset yhdisteet, indusoitunut puolustus, kuusi, liukoiset hiilihydraatit, monoterpeenit, *Ophiostoma,* stilbeenit

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Preface

Numerous people in several institutions have been helpful during the course of this thesis. I acknowledge greatly the help of following persons and institutions. Kim von Weissenberg (currently University of Helsinki), Erik Christiansen and Halvor Solheim (Norwegian Forest Research Institute, Ås) and Susanne Harding (Royal Veterinary and Agricultural University, Copenhagen) guided me years ago as under-graduated student to the fascinating world of the bark beetles associated fungi. They have given valuable advice in early phase and your work has given inspiration during the whole project. This work has been financially supported mainly (1995-1999) by the Graduate School of Forest Sciences, Ministry of Education. Further funds have been provided: Finnish Forest Research Institute; Faculty of Forestry and Centre of Excellence Programme, University of Joensuu; The Finnish Society of Forest Science and Niemi Foundation. I express my thanks for the financial support.

Pekka Niemelä has been supervisor of this thesis. I thank you from encouragement and support during these years. You have been a good company. Esko Valtonen gave valuable advice for statistical analysis for Paper I. Jacques Garcia, Paul Romary, Annie Yart (Institut National de la Recherche Agronomique, Orléans) helped in practical issues during my stay in Orléans. Konkordia Foundation and Emil Aaltonen Foundation supported my visit to France. Co-authors Erkki Annila, Veikko Kitunen and François Lieutier widened my original aims to current form of this thesis. I am also grateful to Erik Christiansen, Markku Keinänen, Maarit Kytö, William Mattson, Vladimir Ossipov, Halvor Solheim and Elina Vapaavuori for improving suggestions concerning manuscripts. Joann von Weissenberg helped to correct the English language and Reija Viinanen made the text lay-out. In last phase, since May 2000 my current immediate superiors at Suonenjoki Research Station, Heikki Smolander and Marja Poteri have been cheering superiors. Thank you from pushing me to write the final dot. Marja also give constructive criticism about the whole bunch. Riitta Julkunen-Tiitto and Michael Wingfield are acknowledged for their work in reviewing the manuscript.

Finally I want to express my gratitude to my family. My parents and parents-in-law have been helpful during these years. My husband Mika Räty, helped with some statistical issues, but mainly I appreciate your efforts to keep my mind and home clean about scientific papers. This thesis is dedicated to our daughter, Vilma, the sparkling light of our lives.

Suonenjoki, 17th September 2002 *Heli Viiri*

Abbreviations

List of abbreviations used in the text, presented in alphabetical order. Journal names are mainly abbreviated according to instructions of the ISI Journal Abbreviation Index, http://www.nal.usda.gov/indexing/lji99/ and the International Organization for Standardization, International Serials Data System, CIEPS Paris 1985, ISBN 2-904938-02-8.

Symbol:	**Description:**
BA_1	cross-sectional area of the current annual ring
BA_1/SA	vigour index, the ratio of the cross-sectional area of the current annual ring and the sapwood basal area at breast height
C-based	carbon-based
CHN analyser	carbon-hydrogen-nitrogen elemental analyser
CNB hypothesis	carbon/nutrient balance hypothesis
C/N ratio	carbon/nitrogen ratio
CBSCs	carbon-based secondary compounds
DBH	diameter at breast height (1.3 m)
de novo	*novus* = new, *de novo synthesis* of organic compound in biochemical reaction
DF, *df*	degree of freedom
e.g.	*exempli gratia,* for example
GC	gas chromatography
GDB hypothesis	growth/differentiation balance hypothesis
ICP-AES	inductively coupled plasma emission spectrometer
in vitro	outside the living organism
MS	mass spectrometry
MT-type	*Myrtillus*-type in the Finnish forest soil-type classification
N	nitrogen
n	sample size
N-based	nitrogen-based
NPK	nitrogen-phosphorous-potassium
P	phosphorous
PAL	phenylalanine ammonia lyase
PP cells	polyphenolic parenchyma cells
SA	sapwood basal area at breast height (1.3 m)
SD	standard deviation of series
s.s.	*sensu stricto*

List of original articles

This thesis is based on the following articles, which are referred to in the text by Roman numerals I–IV. The published articles are reprinted with kind permission of the publishers.

I Viiri, H. 1997. Fungal associates of the spruce bark beetle *Ips typographus* L. (Col. Scolytidae) in relation to different trapping methods. Journal of Applied Entomology 121: 529–533.

II Viiri, H. and Lieutier, F. Ophiostomatoid fungi associated with the spruce bark beetle, *Ips typographus,* in post-epidemic areas in France. (Submitted).

III Viiri, H., Annila, E., Kitunen, V. & Niemelä, P. 2001. Induced responses in stilbenes and terpenes in fertilized Norway spruce after inoculation with blue-stain fungus, *Ceratocystis polonica*. Trees – Structure and Function 15: 112–122.

IV Viiri, H., Niemelä, P., Kitunen, V. & Annila, E. 2001. Soluble carbohydrates, radial growth and vigour of fertilized Norway spruce after inoculation with blue-stain fungus, *Ceratocystis polonica*. Trees – Structure and Function 15: 327–334.

In Study II all stages were planned and carried out by H. Viiri; F. Lieutier assisted in selecting study areas and collecting the beetles. For Papers III–IV E. Annila and P. Niemelä were in charge of organising the field experiment; V. Kitunen was in charge of the chemical analyses and H. Viiri analysed the results and submitted the papers.

Contents

Introduction

Phloem-feeding bark beetles are destructive pests in coniferous forests. They breed sub-cortically in trees, thus utilising a wide range of dead and dying trees to healthy trees. There are about 2,000 beetle (Coleoptera) species in Finnish forests; and about 800 of these species are saproxylic, depending upon dead trees (Siitonen 1998). Only a few bark beetles do attack healthy trees. The Eurasian spruce bark beetle, *Ips typographus* L. (Coleoptera, Scolytidae), is one of these and is a serious and widely distributed pest on Norway spruce, *Picea abies* (L.) Karsten. Adults and larvae live under the thick bark, usually in dying and weakened trees. While constructing breeding chambers and galleries in the phloem, beetles distribute spores of fungi to their hosts. As they grow, fungal hyphae suppress water transportation, cause discolouration of wood and help the beetles to kill trees. Each new generation of beetles transports the fungi to new host trees. All aspects of symbiotic relationship are not well understood. Among these features is that the fungi seems to make host carbohydrates available to the beetles as nutrition.

Many species of fungi have been reported to occur in association with Scolytidae. Most of them belong to the genus *Ceratocystis sensu stricto* (*s.s.*) Ellis & Halstedt, *Ophiostoma* H. & P. Sydow, *Ceratocystiopsis* Upadhyay & Kendrick and anamorph genera such as *Pesotum* Crane & Schoknecht and *Leptographium* Lagerberg & Melin (Rumbold 1931, Mathiesen-Käärik 1960, Upadhyay 1981, Whitney 1982, Solheim 1986, Schowalter and Filip 1993, Okada *et al.* 1998, 2000). This economically important but taxonomically controversial group has been referred to as the "ophiostomatoid fungi" (Wingfield *et al.* 1993). These fungi are adapted for dispersal by insects: elongated ascocarps bear ascospores at the apices of their necks, which may be protected by a gelatinous matrix. The bark beetles transport spores both laterally and in the digestive tract.

A main aspect of the symbiosis between bark beetles and their associated fungi is their joint action to overcome the resistance mechanisms of their host trees. The symbiosis between bark beetles and their associated fungi has been reviewed extensively (Mathiesen-Käärik 1960, Francke-Grosmann 1963, Whitney 1982, Beaver 1989, Paine *et al.* 1997). In several investigations *Ophiostoma bicolor* Davidson & Wells, *Ophiostoma penicillatum*

(Grosm.) Siemaszko, *Ophiostoma piceae* (Münch) H. & P. Sydow, *Ophiostoma piceaperdum* (Rumbold) Arx and *Ceratocystis polonica* Siemaszko have been found to be associates of Eurasian spruce bark beetles (Siemaszko 1939, Solheim 1986, Harding 1989, Krokene and Solheim 1996). *C. polonica* is the most pathogenic of these fungi and when mass inoculated into Norway spruce, is able to kill even healthy trees (Horntvedt *et al.* 1983, Christiansen 1985b). Although *O. piceaperdum* can also invade the phloem and sapwood and disrupt the water-conducting system (Harding 1989), mostly *C. polonica* is thought to play a special role in the bark beetle -host tree interaction (Solheim 1993, Krokene and Solheim 1998).

Exploitation of healthy trees as breeding material and a nutrition base causes inevitable difficulties to bark beetles. Conifers have several constitutive mechanisms for protecting themselves against pests and pathogens, e.g. thick bark, needle waxes and sticky primary resin. Conifers also have induced defensive mechanisms, *e.g.* the hypersensitive reaction against infection by pathogens (Berryman 1969). The pathogen is sealed off from living tissues of the host by a rapidly expanding, controlled necrosis (Berryman 1969, 1972). Several secondary metabolites are antifungal and antiherbivory, thus preventing the growth and reproduction of fungi and insects. After wounding, the content and quantity of the terpenoid compounds, resin acids, monoterpenes and sesquiterpenes in resin change and become more toxic. Similar changes occur in phenolic compounds like stilbene derivatives. In spite of the constitutive and induced responses that work together to protect the tree, in some circumstances fungi and beetles can escape from the entrapping lesion (Berryman 1982). Variation in the qualitative and quantitative production of secondary metabolites is an essential factor in modifying plant-herbivore interactions.

Aims of the study

The main aim of this work was to study interactions between the host tree, the pathogen and its bark beetle vector as well as to identify factors that influence the resistance of Norway spruce to infection by *C. polonica*.

The first section is methodological and considers whether the spruce bark beetles collected (i) individually, (ii) with pheromone traps using an insulating material in the collecting bottles and (iii) in pheromone traps without insulating material differ in frequency and abundance of associated fungi.

The second section includes identification of the ophiostomatoid species associated with *I. typographus* at different populations. The hypothesis was that there are no differences in frequencies of fungi collected at different geographic locations in low and higher population level of beetles.

The third section covers interactions between the host and the pathogen. Changes in the main secondary compounds of Norway spruce, terpenoids and stilbenes, were studied in relation to carbon allocation and to the nutrient status of trees after artificial inoculation with *C. polonica*.

1 Spruce bark beetle-fungi-host tree interaction – a review

1.1 Spruce bark beetle

1.1.1 Damage

Large-scale outbreaks of spruce bark beetles can cause severe damage throughout spruce forests over areas covering tens of thousands of square kilometres. In central Europe severe damage has been recorded since the 1700's. The damage was most serious immediately after the Second World War, when throughout Central Europe about 30 million m^3 of spruce died. During recent years the spruce bark beetle has killed millions of cubic metres of spruce annually *e.g.* in Germany, France, Austria and Japan (Boutte 1993, Klimetzek and Yue 1997). In Japan, the related species *Ips typographus japonicus* (Niijima) is a destructive pest on *Picea jezoensis* (Siebhold and Zucc.) (Yamaoka *et al.* 1997).

In Fennoscandia in 1971–1982 there was an extensive epidemic, which followed windfall and dry summer periods (Austarå *et al.* 1983, Bakke 1983, Worrell 1983, Risberg 1985). In Norway, the damage reached its peak in 1978–1980, when about one million cubic metres of spruce were killed annually (Christiansen and Bakke 1988). In Sweden 1.7–2.8 million m^3 of spruce died during this epidemic. In Denmark, the damage was slight up until the 1960's but drought and windfalls favoured bark beetles' reproduction in the 1970–1980's (Ravn 1985). Also in Finland, the spruce bark beetle is the most damaging scolytid on Norway spruce. However, during the recent period of intensive forest management, bark beetle populations have not risen to high levels. In Finland, compared with the other Nordic countries, both damage and population levels of the spruce bark beetle have been modest (Saalas 1919, 1949, Löyttyniemi and Uusvaara 1977, Löyttyniemi *et al.* 1979, Weslien *et al.* 1989, Valkama *et al.* 1997).

1.1.2 Ecology

As breeding material, spruce bark beetles usually favour weak and dying trees, windfalls and snow breaks. When more suitable breeding material is available, the population size starts to increase rapidly. If the population level is high, the spruce bark beetle can successfully attack even healthy trees (Bakke 1983, Mulock and Christiansen 1986). Damage is considerable because these beetles favour large spruce trees; and once started, an outbreak can continue for several years.

In Fennoscandia the beetles disperse in May–June, when the beetles emerge from their hibernation sites in the forest litter. Small numbers of beetles also overwinter under the bark of felled trees or in the lower trunk of standing trees. Timing of the flight period has been investigated in several studies (Annila 1969, 1977, Bakke *et al.* 1977a). The most significant regulators of flight have been found to be an air temperature and thermal sum. Most individuals become sexually mature and swarming starts when the air temperature reaches +20 °C. In southern and central Finland the dispersal period normally occurs between the end of May and early June, and in northern Finland in the middle of June (Annila 1969, 1977). New generations emerge from trees into the forest in late July or early August. In Fennoscandia and at high altitudes, only one generation is produced per year, while in more favourable areas in southern Europe normally two, or even three, generations occur each year.

Spruce bark beetles have a chemical system of communication that helps them to select suitable host trees and allows the beetles to colonize them effectively (Bakke 1983). The beetles' pheromone system co-ordinates the intensity of the attack, causing rapid aggregation of large numbers of beetles (Bakke *et al.* 1977a, Birgersson *et al.* 1984, Schlyter and Birgersson 1989). In *Ips* species, the male beetles initiate construction of an entrance tunnel and gallery by producing mainly the components of aggregation pheromone (Bakke *et al.* 1977b). In *I. typographus*, the aggregation pheromone is composed of two primary components; methylbutenol is a short-range attractant that promotes landing and entering of holes, while the heavier and less volatile *cis*-verbenol also promotes landing but acts over a longer distance (Bakke *et al.* 1977b, Schlyter *et al.* 1987). Conspecifics of both sexes are attracted to the site; males initiate their own nuptial chambers, and one to four females arrive to the completed chambers to mate with the resident male (Christiansen 1988). After mating, the female starts to excavate

longitudinal egg galleries in the phloem and the male helps remove from the gallery the dust produced by boring.

The pheromone production of bark beetles is linked to the secondary metabolism of the host tree. The resin monoterpenes can have contrasting effects as bark beetle repellents and as pheromone precursors. *Cis*-verbenol is synthesized by hydroxylation of the exogenous monoterpene of the tree, (-)-α-pinene. The synthesis of this compound is regulated by the availability of the precursor (-)-α-pinene (Ivarsson 1995). Since spruce produces (-)-α-pinene to defend itself, the beetles can produce *cis*-verbenol and attract more individuals to the tree (Birgersson 1989, Ivarsson 1995). The spruce bark beetle can synthesize methylbutenol *de novo* via mevalonate, independently of precursors from the host tree (Lanne *et al.* 1989). In addition, yeasts associated with spruce bark beetles can convert *cis*-verbenol to verbenone (Leufvén *et al.* 1984). Furthermore, verbenone and ipsenol act as antiaggregation pheromones regulating breeding density in trees (Bakke 1981, Birgersson *et al.* 1984).

During gallery construction, many bark beetles transport various micro-organisms to the phloem and cambium: yeasts, bacteria and fungi that help the beetles establish galleries and begin to oviposit as well as aiding digestion (Whitney 1982, Leufvén and Nehls 1986, Furniss *et al.* 1990). In some bark beetles slimy secretions may be produced to preserve ascospores and conidia from desiccation and UV-light (Dowding 1969). Many species of bark beetles have special organs, known as mycangia, in which spores are transported. In general, the mycangia have a similar basic structure: tubes, pouches, cavities or pits associated with glandular cells (Beaver 1989). The spruce bark beetle, however, does not have mycangia and it transports fungal spores mainly laterally on the posterior half of the pronota and in pits scattered over most of the surface of the elytra. Small numbers of spores are also transported in the digestive tract and on phoretic mites associated with the beetles (Moser *et al.* 1989, Furniss *et al.* 1990). Either *I. typographus* has slimy excretions in the pits, where the spores occur or the spores may be covered with wax (pers. comm. E. Christiansen and H. Solheim). Thousands of ascospores and conidia may be found on the surface of each beetle, but the number of spores transported by different individuals varies greatly. Neither ultrasonic cleaning nor chemical sterilizing agents have successfully eliminated fungal spores from adult beetles (Harding 1989, Furniss *et al.* 1990).

1.2 Associated fungi

1.2.1 Ecology

Ophiostoma and *Ceratocystis* fungi are well adapted for dispersal by insects: elongated ascocarps bear ascospores on the apices of their necks; these ascospores may be released in a slimy matrix. The fruiting structures of the fungi are formed in beetle galleries and under bark flaps on the phloem. Since spores lose their vitality and dry in sunshine, rapid insect dispersal guarantees reliable inoculation of the fungi to a suitable habitat (Dowding 1969). In addition, the conidia of ophiostomatoid fungi may accumulate in sticky drops at the apex of conidiophores. The cirri of some ophiostomatoid species disperse in conifer resin rather than only in water (Whitney and Blauel 1972). According to Wingfield *et al.* (1993), species with short ascomatal necks tend to have long ascospores and *vice versa*. In spite of that, variation in the length of necks or the presence of ostiolar hyphae does not correspond to the probability of successful dispersal at the species level.

Many ophiostomatoid fungi associated with bark beetles are highly pathogenic and also cause sap-stain, which is a grey, black or bluish discoloration of sapwood caused by the optical effect of pigmented fungal hyphae (Wingfield *et al.* 1993). It is quite often referred to as blue-stain; thus the word "blue" for describing the discoloration can in many cases be misleading. There is a continuum from truly pathogenic sap-staining fungi that occur in living trees to pathogenic fungi that grow on weakened trees to truly saprobic fungi that utilise dead trees (Wingfield *et al.* 1993). Typically, discoloration is spread over a wide area of the stem, (more rapidly longitudinally than radially) causing deep staining throughout the sapwood. Hyphae grow mainly in tracheids and ray parenchyma cells, preventing water transport, and can kill living tissues far from the beetle galleries (Wong and Berryman 1977, Horntvedt *et al.* 1983). No actual staining of the cell walls occurs (Wingfield *et al.* 1993). Despite the fact that tangential growth and radial growth in the xylem ray system are minor, fungi can reduce the commercial value of the lumber considerably. However, the main harmful effects of pathogenic ophiostomatoid fungi are visible in the forest when trees are dying.

Common associates of the spruce bark beetle during a beetle epidemic and immediately following are *C. polonica, O. bicolor, O. penicillatum* and *Pesotum* species (Table 1). These species are frequent in the area of visible staining (Solheim 1986, 1992a,b). When the population level of beetles has been low, the same species have occurred; but the frequency of *C. polonica* has been

lower than during epidemics (Harding 1989, Solheim 1993). It has been proposed that *C. polonica* may be replaced by other species during periods when the population level is low and the beetles infest dead trees and timber (Solheim 1993). During epidemics, however, living trees are attacked and the frequency of *C. polonica* may increase (Solheim 1993, Wingfield *et al.* 1993). *C. polonica* is the most aggressive species, being able in inoculation experiments to invade sapwood and kill healthy trees (Horntvedt *et al.* 1983, Christiansen 1985b, Solheim 1988, Kirisits 1998, Krokene and Solheim 1998). It tolerates low oxygen levels and grows extensively in the phloem and sapwood (Solheim 1991), while other ophiostomatoid fungi cannot grow well in such low levels of oxygen.

Table 1. Ophiostomatoid species associated with the spruce bark beetle *I. typographus* with their anamorphs.

Species	Anamorph	Reference
Ceratocystiopsis minuta (Siemaszko) Upadhyay & Kendrick	*Hyalorhinocladiella*	3,4,6,9,11-15
Ceratocystis polonica Siemaszko	*Thielaviopsis*	1,3-7,11-15
Ophiostoma ainoae Solheim	*Pesotum*	3,11-15
O. bicolor Davidson & Wells	*Hyalorhinocladiella*	1,3-5,9,11-15
O. cainii (Olchowecki & Reid) Harrington	*Pesotum*	3
O. cucullatum Solheim	*Pesotum*	3,4,11,15
O. japonicum Yamaoka & M.J. Wingfield	*Pesotum*	15
O. flexuosum Solheim	*Sporothrix*	3,11
O. penicillatum (Grosm.) Siemaszko	*Leptographium*	1-9,11-15
O. piceae (Münch) H. & P. Sydow	*Pesotum*	2-5,7-9,11-15
O. piceaperdum (Rumbold) Arx = *O. europhioides* (Wright & Cain) Solheim	*Leptographium*	1,3,4,11-15
O. piliferum (Fries) H. & P. Sydow	*Sporothrix*	9
O. pluriannulata (Hedgcock) H. & P. Sydow	*Sporothrix*	7
O. stenoceras (Robak) Melin & Nannfelt = *O. albidum* Mathiesen-Käärik	*Sporothrix*	7
O. tetropii * Mathiesen	*Sporothrix*	6,9,11,13

*Note: Dubious validity of species according to Jacobs and Wingfield (2001).

References: 1=Davidson *et al.* 1967, 2=Grosmann 1931, 3=Harding 1989, 4=Kirisits 1996, 5=Krokene and Solheim 1996, 6=Mathiesen 1951, 7-8=Mathiesen-Käärik 1953,1960, 9=Savonmäki 1990, 10=Siemaszko 1939, 11-14=Solheim 1986, 1992a,b, 1993 and 15=Yamaoka *et al.* 1997.

1.2.2 Taxonomy

Most of the fungi associated with bark beetles that cause discoloration belong to the ascomycetes or the fungi imperfecti. Important and economically significant associates belong to the family Ophiostomataceae and in particular to the genera *Ceratocystis* and *Ophiostoma* (Table 1). *Ceratocystis*, *Ceratocystiopsis* and *Ophiostoma* are differentiated according to properties of ascospore morphology, development of the ascomatal centrum, carbohydrate content of the cell walls, conidial stages and conidia. Order, family and several species have been a source of taxonomic controversy. The families *Ceratocystis s.s.*, *Ceratocystiopsis* and *Ophiostoma* have generally been accepted. Most species in the genus *Ceratocystiopsis* share common characteristics with species in *Ophiostoma*, and it has been proposed that the species form a monophyletic group (Wingfield *et al.* 1993, Viljoen *et al.* 2000). Many bark beetle associates formerly considered to be in the genus *Ceratocystis* are now placed in the genus *Ophiostoma* (Wingfield *et al.* 1993, Hawksworth *et al.* 1995, Viljoen *et al.* 2000). The main species in this thesis, *C. polonica*, was originally described as *O. polonicum* and has lately been transferred to *Ceratocystis* (Visser *et al.* 1995, Harrington *et al.* 1996). Furthermore, based on the morphology of the perithecia, Jacobs *et al.* (2000) concluded that *O. europhioides* and *O. piceaperdum* are indistinguishable thus supporting the synonymy *O. piceaperdum* proposed by Upadhyay (1981). In the interest of consistency, throughout this review *O. europhioides* is called *O. piceaperdum*.

The members of the genus *Ceratocystis s.s.* contain no cellulose or rhamnose in the cell walls, and traditionally the asexual stage has been *Chalara* Corda (Weijman and De Hoog 1975, Upadhyay 1981, De Hoog and Scheffer 1984). Recently Paulin-Mahady *et al.* (2002) transferred anamorphic *Chalara* species to the genus *Thielaviopsis* Went. Within these *Ceratocystis* species, antibiotic cycloheximide in the growth media prevents growth, which has been used as a taxonomic characteristic of the genus (Harrington 1981). Other characteristics are darkly pigmented (nearly black) perithecia and hyaline ascospores that vary in shape but are not falcate (De Hoog and Scheffer 1984). *Ophiostoma* and *Ceratocystiopsis* species have cellulose and rhamnose in the cell walls, which may be the reason for their tolerance to cycloheximide (Harrington 1981). The perithecia are almost glutinous, and the ascospores vary in shape. *Ceratocystiopsis* species are otherwise similar to *Ophiostoma*, but the ascospores are elongated or falcate and are

surrounded by a hyaline, gelatinous ascospore wall (previously defined as a sheath) (Upadhyay and Kendrick 1975, Upadhyay 1981, De Hoog and Scheffer 1984).

Ophiostomatoid fungi can have several asexual forms or, in some species, only the asexual form is known. The family Ophiostomataceae has contained as many as 16 asexual forms (Wright and Cain 1961, Upadhyay and Kendrick 1975, Upadhyay 1981, Wingfield *et al.* 1993). Asexual forms are classified according to the morphology of the conidiophores or by the form of the conidia. The conidiophores can be mononematous like *Leptographium* or form synnemata like *Pesotum*. Synnematous anamorphs of *Ophiostoma* species were quite recently placed in the genus *Graphium*, although *Graphium* species are considered to be anamorphs of the Microascales (Okada *et al.* 1998). However, the synnematous anamorphs of *Ophiostoma* species are phylogenetically unrelated to *Graphium s.s.*, and should be currently referred to the anamorph genus *Pesotum* (Okada *et al.* 2000). The genus *Verticicladiella* is considered to be a synonym for *Leptographium* (Wingfield 1985, Schowalter and Filip 1993). Most of the asexual forms of the *Ophiostoma* species can be classified to the genera *Pesotum* (Okada *et al.* 1998, 2000), *Leptographium, Hyalorhinocladiella* or *Sporothrix* (Harrington 1987, Hausner *et al.* 2000).

The taxonomy of the family Ophiostomataceae and its genera still needs clarification. In recent years, several new fungi that cause wilting and discolouration on trees have been described. Unknown species complexes probably exist even in fungi that have worldwide distribution and a wide host range, for example, *C. fimbriata*, for which related species, *C. albofundus* Wingfield, De Beer and Morris have lately been found (Wingfield *et al.* 1996). Morphological similarities in teleomorph and anamorph structures of phylogenetically distinct *Ceratocystis* and *Ophiostoma* have apparently developed as an adaptation to an insect-associated habitat (Visser *et al.* 1995, Hausner *et al.* 2000).

1.3 Host tree

1.3.1 Vigour

Measurements of tree vigour have been used to determine the risk of attack by bark beetles and as a substitute for the term "resistance" (Waring and Pitman 1983, Mulock and Christiansen 1986). The tree vigour index (BA_1/SA) is defined as the ratio of the cross-sectional area of the current annual ring (BA_1) to the

sapwood basal area (SA) at breast height (Waring *et al.* 1980, Münster-Swendsen 1987). The basic assumption is that good growth generates high vigour. The index is based on the pipe-model theory and on the assumption that the relationship between the cross-sectional sapwood area and the weight or area of leaves supported by the conducting sapwood within a tree species is linear.

The number of beetle attacks, their distribution on the trunk and their timing determine the effectiveness of the bark beetle - fungi association complex in overcoming the resistance of a tree (Berryman 1972, 1982, Raffa and Berryman 1983, Christiansen 1985a,b). The spruce bark beetle can colonise standing healthy Norway spruce trees if the number of attacking beetles is large enough to overcome the resistance of the trees. In experiments, attacks of 150–200 beetles or artificial inoculations of *C. polonica* have killed the trees (Christiansen and Horntvedt 1983, Christiansen 1985b). During an epidemic in nature, hundreds or even thousands of spruce bark beetles can attack a single mature tree. Bark beetles both co-operate in overwhelming the host defense due to pheromone mediated mass-attack and simultaneously compete for the available resources.

Stand and climatic conditions often reduce plant assimilation and may consequently lower a tree's ability to resist the attack of bark beetles and associated fungi. Changes in herbivore abundance have often been correlated positively with unfavourable environmental factors. On the other hand, the ability of a tree to defend itself is linked to its overall vigour and to the amount of carbohydrates that can be used as a source of energy for synthesising defensive compounds (see reviews: Berryman 1972, Christiansen *et al.* 1987, Paine *et al.* 1997). This is especially important in mature conifers, which have reduced ability to replace damaged structures.

1.3.2 Constitutive defense

Conifers protect themselves against attack by bark beetles and associated fungi with constitutive defense, based mainly on pre-formed primary resin, and by production of secondary resin in the induced wound response. Constitutive defense is found especially in trees with well-developed pre-formed resin duct systems, such as *Pinus*. Primary resinosis is a continuous defense system against generalists. It is particularly important during the early phases of the beetle-fungus colonization as an agent that cleanses the wound (Berryman 1972) and enables activation of the induced defense.

Primary resin is under turgor pressure in the vertical and horizontal resin ducts and blisters and depends on the water potential of the tree. The resin starts to exude when the tree is wounded, *e.g.* by the boring activity of beetles. The flow and the crystallization of the primary resin are sometimes sufficient to deter beetles soon after they initiate construction of galleries (Reid *et al.* 1967, Hodges *et al.* 1979). In addition, pre-formed primary resin inhibits the growth of ophiostomatoid fungi (Cobb *et al.* 1968). During a mass attack by bark beetles the resin system is often exhausted due to simultaneous exudation of resin in separate wounds that lead to successfully constructed galleries. In spruce, constitutive resin is carried primarily in vertical resin ducts in the bark, and the amount of resin content depends mainly on the storage capacity of the duct system (Christiansen and Bakke 1988). Thus, in Norway spruce exudation of primary resin varies considerably between individual trees (Christiansen and Horntvedt 1983). In addition to resin, lignified stone cell masses (lignin) provide an important pre-formed system of defense in living trees by preventing both construction of bark beetle galleries and oviposition (Wainhouse *et al.* 1998).

1.3.3 Induced wound response

The induced wound response plays an essential protective role in conifers that lack a well-developed pre-formed system of resin ducts, *e.g.* species in the genus *Picea*. The induced wound response has been thought to protect trees against host-adapted pests and pathogens. Fungal cell-wall components and bark beetle feeding elicit a metabolically active induced wound response in the phloem and sapwood (Miller *et al.* 1986, Lieutier and Berryman 1988). Living cells in the reaction zone die and form a necrotic area, thus preventing fungal nutrition. Berryman (1969) described this controlled necrosis as the hypersensitivity reaction which is an active metabolic process affected by the physiological vigour of the tree. Soluble nutrients and carbohydrates are mobilized from tree storage reserves to produce toxins in tissues near the point of infection; the amount of soluble carbohydrates decreases and the content of secondary metabolites increases and becomes more toxic (Reid *et al.* 1967, Wright *et al.* 1979, Cook and Hain 1985, 1987, Delorme and Lieutier 1990, Raffa and Smalley 1995). Thus the reaction zone fills with resinous and C-based compounds. Monoterpenes, sesquiterpenes, resin acids and phenolic compounds repel beetles and inhibit the growth of fungi or bark beetle larvae (Shrimpton and Whitney 1968, De Groot

1972, Bordasch and Berryman 1977, Raffa *et al.* 1985, Bridges 1987, Woodward and Pearce 1988, Delorme and Lieutier 1990, Solheim 1991, Klepzig *et al.* 1996, Lindgren *et al.* 1996, Evensen *et al.* 2000). Secondary resin, as well other secondary metabolites, is produced by ray and phloem parenchyma cells. In addition, callus and wound periderm develop rapidly in tissues surrounding the wounded area (Reid *et al.* 1967, Berryman 1969, Wong and Berryman 1977). A reaction zone is formed to separate wounded tissues from healthy wood.

Fungal inoculation tests and resin toxicity tests with bark beetles have shown that the rate of accumulation and the concentration of secondary metabolites in the reaction zone are crucial for successful protection. The components of primary and secondary resin vary qualitatively and quantitatively between individual trees, species, age, season and type of resin (Russell and Berryman 1976, Raffa and Berryman 1982, 1983, Toscano Underwood and Pearce 1991, Lindberg *et al.* 1992). In general, non-host resins are more toxic to insects and fungi than resins from the host species; and the induced resins are more toxic than the pre-formed resins. Formation of the reaction zone and terpene synthesis represents two independent activities during the wound response, necrosis proceeding more rapidly than terpene synthesis (Raffa and Berryman 1982). Thus, the fungus is first contained by the removal of essential nutrients from the entry site, and only secondarily by resinosis (Wong and Berryman 1977, Raffa and Berryman 1982, Christiansen and Ericsson 1986).

In woody plants the induced wound response is considered to be a non-specific response to wounding. However, it has been detected that concentrations of monoterpenes increase as the virulence of the fungal species increases (Cook and Hain 1985, Popp *et al.* 1995). On the other hand, it is a competitive advantage for many pathogenic fungi to tolerate stilbenes better than non-pathogenic species do (Hart 1981). When inoculated into a tree, a pathogenic fungus causes a typical reaction zone in the phloem, the size of which depends on variation in fungal growth, virulence or elicitor production.

The phloem of Norway spruce contains so-called polyphenolic parenchyma cells (PP cells) and traumatic resin ducts. These cells and ducts react to fungal inoculation and wounding, which indicate involvement of both constitutive and inducible defense responses (Franceschi *et al.* 1998, 2000). Pre-formed and induced wound responses are separate, but overlapping, dynamic phases of wound reaction; the pre-formed reaction consists mainly of processes ending up to visual symptoms, and the induced wound response consists more of

biochemical changes at the cell and tissue level. Induced defense, in particular, is energy-demanding response involving *de novo* synthesis of secondary compounds and new tissue. Despite the fact that the constitutive and induced wound responses work together to protect the tree, in favourable circumstances fungi can escape from the entrapping reaction zone.

2 Methods

(For details, please refer to the original articles I–IV)

2.1 Isolation and identification of fungi

Living beetles were inoculated into fresh logs in order to isolate the associated ophiostomatoid fungi (**I, II**). The inoculation method was originally described by Wright (1933) and has been used widely in corresponding studies. Beetles were placed with forceps into the hole made by a cork-borer, and the bark plug was then reinserted. Empty holes made by a cork-borer but without beetles were used as control inoculations. The aim of the control inoculations was to detect fungi that had become established in the phloem or bark before inoculation or invaded trees during the inoculation phase as contamination. To kill air-dispersed spores, the surface of the logs was sterilized with ethanol before inoculations. After 3 and 4 weeks incubation, samples were taken from bark, phloem or sapwood.

Malt-agar media suitable for most ascomycetes were used in culturing. To promote fungal sporulation, wooden chips were occasionally added to the growth media. The growth media were not optimised for the growth demands of any special fungi, because the aim was to isolate all possible ophiostomatoid fungi. Identification concentrated on ophiostomatoid species, and thus other species that did not produce teleomorph were mostly ignored. The only anamorph of *Ophiostoma* and *Ceratocystis* species identified to species level was *Leptographium penicillatum* Grosmann. There are several discrepancies among the anamorph descriptions, so in many cases identification would have been uncertain without a teleomorph (Wingfield *et al.* 1993). Following cultures from the collections of the Norwegian Forest Research Institute were used as reference material in identification: *O. penicillatum* (80–91/54), *C. polonica* (80–53/7), *O. piceae* (80–92/34), *O. europhioides* (80–91/9), *O. ainoae* (80–85/37), *O. tetropii* (80–113/9) and *Graphium* sp. (80–52/24).

2.2 Fungal inoculations in living trees

Low-density inoculations were made in a *Myrtillus*-type mature spruce forest in Vesijako, southern Finland (**III, IV**). Four plots were marked, and in May 1993 fertilization treatments (N, P and NPK) were applied to the plots to manipulate the defensive potential of the trees. The control treatment was a non-fertilized plot. From each plot, 30 trees that were free of visible wounds, a total of 120 trees, were selected. In June 1994 a Finnish culture (origin Tuusula) of *C. polonica*, on malt-agar in petri dishes was inoculated with a cork-borer into ten trees per fertilization treatment. Each wounded tree received four inoculations, one at each of the cardinal points of the compass, at 1.3 m above ground level. For mechanical wounding, the trees were injured with a cork borer in the same way as inoculation, but without the fungus. Ten trees per fertilization treatment were wounded mechanically.

After a two-month incubation period around each fungal inoculation site, phloem samples were taken with the borer to the level of the cambium. One sample was collected from the distal ends of the visible reaction lesion (later called the "far" samples) and one each from the areas immediately above and below the site of inoculation (later called the "near" samples). Two samples were taken from near the site of mechanical wounding, one above the inoculation site and another below the inoculation site. Unwounded phloem from non-bored trees was used as the unwounded control.

2.3 Chemical analyses

Chemical analyses were conducted in the Central Laboratory of the Finnish Forest Research Institute, Vantaa Research Centre. Phloem samples were ground in liquid nitrogen and analysed for their terpene, stilbene and carbohydrate composition. To obtain sufficient amounts of compounds for gas chromatography (GC) analysis, organic solvents were used for extraction. Stilbenes, carbohydrates, mono- and sesquiterpenes were analysed in a GC mass spectrometry (MS) system with a capillary column. The degradation of stilbene compounds was reduced by silylating the samples. Stilbene glycosides were quantified according to the response of rhapontin, and stilbene aglycones were quantified by using the response factors of diethyl stilbestrol and resveratrol.

Monoterpenes were identified using the retention and mass spectral data of authentic model compounds. Sesquiterpenes were identified according to the method of Pohjola (1993) and

quantified according to the response factor for caryophyllene. Due to the complexity of the compounds analysed, both retention and MS data were used for identification. In identification of compounds previously published data were also used (Mannila 1993, Pohjola 1993). The enantiomers of chiral monoterpene hydrocarbons were not separated. For quantification of compounds, calibration with both internal and external standards was used. When possible, commercial substances were used as reference compounds.

The needles of the same trees were used for analysing nutrient concentrations with an inductively coupled plasma emission spectrometer (ICP-AES). The total C and N in needles were determined by dry combustion with a CHN analyser.

3 Results and discussion

3.1 Fungal flora

Species *Ceratocystis polonica*, *O. ainoae*, *O. bicolor*, *O. piceaperdum*, *O. penicillatum*, *O. piceae* and *O. tetropii* and asexual forms of *Leptographium* spp. and *Graphium* spp. were found to be associated with spruce bark beetles in Finland (Paper **I**). These species correspond to the fungal flora previously found to be associated with galleries of spruce bark beetle in southern Finland (Savonmäki 1990). In addition, *C. polonica*, *O. ainoae* and *O. piceaperdum* were found as new species. Furthermore, in France were found *C. minuta* and *O. cucullatum* Solheim. The frequency of *C. polonica* was lower in Finland and France than in Norway (Krokene and Solheim 1996). Whether the frequency of pathogenic *C. polonica* as an associate of spruce bark beetle would be more common during an epidemic, could not be clearly answered here. Nevertheless, low frequency of associated pathogenic species is not contradictory to low level of damage in Finland. In French study areas, the spruce bark beetle population were in the post-epidemic phase. Also elsewhere, the high frequency of *C. polonica* has been sporadic and irregular (Harding 1989, Kirisits 1996, Yamaoka *et al.* 1997, Kirisits *et al.* 2000).

In French isolations *C. minuta* was frequent, but it was not detected in Finnish isolations. Lack of *C. minuta* in the Finnish samples may be partly due to difficulties identifying the mixed unsporulated strains (**I**). On the other hand, *C. minuta* perithecia were abundant and easy to identify from the French primary isolations containing even several species (**II**). Also elsewhere the association of *C. minuta* with *I. typographus* has been inconstant (Solheim 1986, Kirisits 1996, Kirisits *et al.* 2000).

Previously no specific importance has been ascribed to *O. piceaperdum*, but my results suggest that the role of this species may vary (**I, II**). *O. piceaperdum* is frequently found in Denmark and Austria, invading the sapwood of Norway spruce and disrupting the water-conducting system (Harding 1989, Kirisits 1996). It is not able to grow as rapidly as *C. polonica* (Harding 1989), but it causes long broad lesions in the phloem (Kirisits 1996, 1998). Results concerning the ability of *O. piceaperdum* to cause sapwood discoloration are conflicting; in Denmark the

species has caused as extensive desiccation as *C. polonica*, but in Austria the desiccation observed after artificial inoculation was slight (Kirisits 1998).

O. piceaperdum has been found in several conifers in Europe (Davidson *et al.* 1967) and in North America (Wright and Cain 1961, Davidson and Robinson-Jeffrey 1965). Recently the species was found to be a constant associate of the Douglas-fir beetle, *Dendroctonus pseudotsugae* Hopkins (Solheim and Krokene 1998). Because of the revised status of *O. europhioides* and *O. piceaperdum*, the pathogenicity and distribution of the *O. piceaperdum* complex needs further investigation in different host trees throughout its broad geographical range.

O. penicillatum is frequently associated with *I. typographus* (**I, II**, Grosmann 1931, Siemaszko 1939, Mathiesen-Käärik 1953, 1960, Harding 1989) and has been isolated from the phloem near visible stains (Solheim 1986, 1992a). *O. penicillatum* does not grow deep into the sapwood, and thus it has been speculated that this species is not able to kill a tree without the beetle (Horntvedt *et al.* 1983, Solheim 1988, Harding 1989). The large internal variation in morphological characters supports the expectation that the pathogenicity of this species is variable (Mathiesen-Käärik 1953, 1960).

O. bicolor occurs with *C. polonica* in the beetle galleries, especially in the early phase of attack (Solheim 1986, Harding 1989). It is a fast growing species (Solheim 1986,1991); but not, according to the inoculation experiments, as aggressive as *C. polonica* (Solheim 1988, Kirisits 1998). Most likely *O. bicolor* can overcome the resistance of a tree with other fungi, but alone it is not able to kill a healthy tree. *O. bicolor* has been isolated from *Ips*-infested spruce both in Europe and in western parts of North America.

Among the ophiostomatoid species, *O. bicolor*, *O. piceaperdum* and *C. minuta* were easy to identify. In the Finnish isolates of *O. piceaperdum*, perithecia formation was very abundant and stable. There were no signs of degeneration that might have influenced its frequency of occurrence. French isolates produced noticeably less perithecia, thus suggesting intra-specific variation. However, the ability of *O. piceaperdum* to produce perithecia seemed to be more stable than that of *e.g. O. bicolor* (**I, II**). *C. polonica*, *O. ainoae* and *O. bicolor* degenerated rapidly after two transfers, producing only sterile mycelia. Degeneration of axenic cultures and loss of pathogenicity has also been noticed by Kirisits (1996) and Krokene and Solheim (2001). In old cultures, after several months incubation and storage at +4 °C, *O. ainoae* produced few perithecia (**I**). *O. piceae* colonies

can also be hard to maintain in normal condition on artificial media, and some colonies do not form perithecia (Davidson 1953). Here on malt-agar some *O. piceae* colonies formed only light brown sectors or grey dots with few coremia and no perithecia. It has lately been reported that the *O. piceae* complex forms a monophyletic group of nine recognized insect-dispersed species, delimited by synnemata morphology, growth rate, mating reactions and sequences of the internal transcribed spacer (ITS) region of the rDNA operon (Harrington *et al.* 2001).

The large numbers of *Leptographium* sp. can be partly ascribed to the ability of *O. penicillatum* to degenerate on artificial growth media when retained for long periods (**I, II**). After several transfers the species produces few perithecia and only occasionally (Davidson *et al.* 1967). According to Kendrick's (1962) extensive review of *Leptographium* species, the *Leptographium* sp. found in study (**I**) appears to be very similar to *L. penicillatum*. *Leptographium* spp. may contain asexual stages of both *O. penicillatum* and *O. piceaperdum*, so asexual stages were not used as the main characteristic in identification. All synnematous anamorphs were included in the group of *Graphium* spp. in Paper **I** and *Pesotum* spp. in Paper **II**, which group probably includes several different species since there was great variation in width, length and colour of the synnemata.

Some species produce few or no perithecia on artificial growth media, and some reproduce more abundantly in wood (Furniss *et al.* 1990). Most species of *Ceratocystis* and *Ophiostoma* vary widely in colony growth, formation of asexual and sexual structures and sporulation, which makes it difficult to produce sexual stages on artificial media. Ophiostomatoid fungi need adequate nutrients and high C/N ratios to produce perithecia on artificial growth media (Mathiesen-Käärik 1960). Progressive sub-culturing favours vegetative growth at the expense of reproductive structures. Moreover, after two or three transfers (**I, II**) some species produce only sterile mycelia.

When fungi have been isolated from attacked trees, inoculated logs or directly from beetles, significant differences have been detected in the fungal flora (Yamaoka *et al.* 1996, Solheim and Krokene 1998). The most reliable method for identifying ophiostomatoid fungi is to isolate them from recently hatched insects. However, after hibernating in the ground, dispersing beetles are covered with soil microbes. Here inoculations of insects (**I, II**) into wooden logs prevented mostly other fungi and microbes from affecting isolation of associated fungi. On the other hand, the isolation technique used here favour pathogenic fungi. Fungi isolated from control inoculations had

become established in the phloem or bark before the inoculation or invaded trees during the inoculation phase as contaminants. Some of the same species, such as *Nectria* sp., were isolated from the true inoculation. In the control inoculation there might have been fungi belonging to the genus *Ophiostoma, e.g. O. piceae* or *O. piliferum*, which can also disperse by air. But surface sterilization of logs (**I, II**) was enough to kill air-dispersed spores. Furthermore, there was no evidence of cross-contamination from other beetles due either to the presence or to the abundance of fungal species when fungi were isolated from beetles collected with different methods (**I**).

Morphological plasticity, conidia production for partitioning conidial stages, and degeneration of conidiophores on artificial growth media during storage and subculturing have led to large numbers of conidial stages in ophiostomatoid fungi. Ultrastructural studies and ribosomal DNA sequencing have been used to explore species characteristics and taxonomic relationships within the genus and between genera (Van Wyk and Wingfield 1990, 1993, Jacobs *et al.* 1996, Hausner *et al.* 2000, Okada *et al.* 2000, Harrington *et al.* 2001, Paulin-Mahady *et al.* 2002). Genera that are indistinguishable with light microscopy may differ in ultrastructural morphology, and phylogenetic and some ascosporic characteristics observed in light microscopy studies can even be misleading. These aspects must be taken into account when these results are considered (**I, II**). To clarify the discrepancy between the results of different investigations, the pathogenicity of different strains of *C. polonica*, *O. penicillatum* and *O. piceaperdum* needs to be tested.

3.2 Induced responses and allocation of resources to defense

Inoculation of *C. polonica* caused extensive lesions around the inoculation site varying in length from 0.5 to 38 cm. Changes in CBSCs were more pronounced with fungal inoculation than with fertilization treatments (**III**). The further lesion formation and induced response had progressed (wounding, fungus inoculation), more total soluble carbohydrates were utilized to prevent fungal invasion (Figures 1–3). It has been proposed that the induced phenolic response of Norway spruce phloem consists of activation of the phenolic pathway, finally leading to production of tannins and insoluble polymers (Brignolas *et al.* 1995, 1998). Present results agreed with these previous findings; detection frequencies of stilbene glycosides decreased in phloem inoculated with fungi

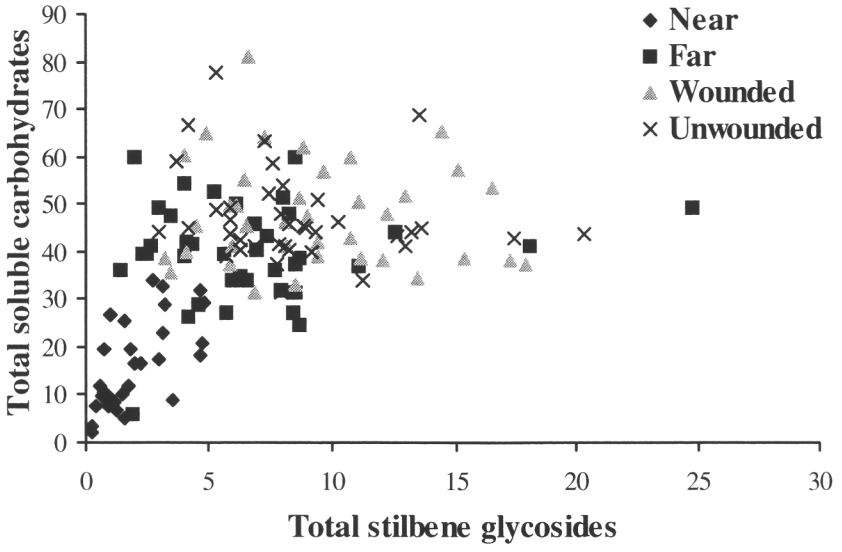

Figure 1. Relationship between total soluble carbohydrates and total stilbene glycosides (μg mg^{-1} fresh phloem) in Norway spruce. For sampling and compounds see Papers **III, IV**. (Near, Wounded, n=38; Far, Unwounded, n=39).

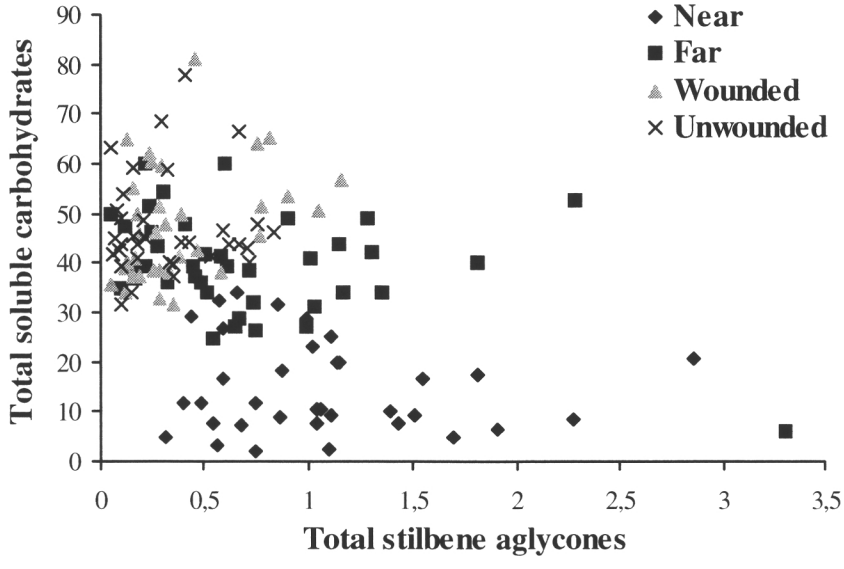

Figure 2. Relationship between total soluble carbohydrates and total stilbene aglycones (μg mg^{-1} fresh phloem) in Norway spruce. For sampling and compounds see Papers **III, IV**. (Near, Wounded, n=38; Far, Unwounded, n=39).

but remained high or at initial concentrations in unwounded phloem (see Fig. 3 in Paper **III**). However, in this study the corresponding aglycones were also detected in small amounts near the point where the fungus had been inoculated. As suggested by the results of (**III**) and those of Woodward and Pearce (1988a), the stilbene aglycones might play a more central role in fungus-challenged tissues than the corresponding glycosides do. Thus the newly synthesized stilbenes might be incorporated into stilbene aglycones and partly into tannins (Figures 1–2). This could explain why in the studies of Brignolas *et al.* (1995, 1998) stilbene glycosides accumulated at higher levels in the susceptible clone than in the resistant clone. However, the results of Evensen *et al.* (2000) contradict those of Brignolas *et al.* (1995, 1998) and do not support stronger induction of the flavonoid pathway than of the stilbene pathway.

Changes in glycosylation of the stilbenes may be located in the PP cells on the basis of their high phenolic and PAL (phenylalanine ammonia lyase) content and to dynamic changes in the cells after wounding (Franceschi *et al.* 1998, 2000, Nagy *et al.* 2000). PAL, a key enzyme in phenolic synthesis, is also present in ray parenchyma cells. The PP cells are the most abundant living cells in the secondary phloem and are thus the most probable site of PAL synthesis. In Norway spruce the terpenoids are stored mainly in the resin ducts, whereas phenolic compounds and PAL are stored in the vacuoles of PP cells (Franceschi *et al.* 1998, Krekling *et al.* 2000). Release of phenolics or metabolites from the vacuole phenolics and traumatic formation of resin ducts provides inducible and sustained release of defensive compounds away from the initial site of wounding or invasion (Franceschi *et al.* 1998, 2000, Krekling *et al.* 2000, Nagy *et al.* 2000). In this study, the accumulation of terpenes near the inoculation point was extensive; the total concentration of terpenes was almost 100 times higher than that in unwounded trees (**III**). Due to the large volume of accumulated terpenoids near the inoculation point (Figure 3), in addition to *de novo* energy-demanding biosynthesis at the site, it is possible that photosynthates were also translocated.

The relationship between host resistance and carbohydrate status in the phloem may be more complex than a simple source and sink relationship. CBSCs cannot be synthesized without substrate, but the presence of substrate does not necessarily lead to high synthesis level of CBSCs. Variation in the responses of CBSCs to fertilization might also be caused by differences in their biosynthesis (Haukioja *et al.* 1998, Koricheva *et al.* 1998) or the storage, transport or maintenance of defenses (Gershenzon 1994). Sesquiterpenes and triterpenes are biosynthesised via the

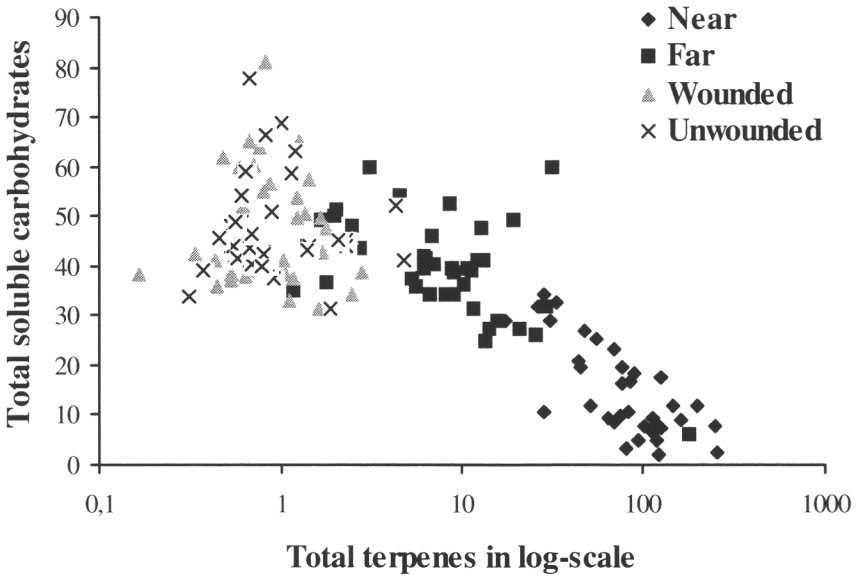

Figure 3. Relationship between total soluble carbohydrates and total terpenes (μg mg^{-1} fresh phloem) in Norway spruce. For sampling and compounds see Papers **III, IV**. (Near, Wounded, n=38; Far, Unwounded, n=39).

mevalonic acid pathway and monoterpenes, diterpenes and tetraterpenes are biosynthesised via the pyruvate-glyceraldehyde-3-phosphate pathway. Meanwhile, phenylpropanoids and defense proteins are synthesised via the same pathway and share a common precursor, PAL. Protein-pathway may compete for excess carbon out of the reach of phenolics, while the mevalonic acid and pyruvate-originated pathways guarantees more "a private" synthesis routes for terpenoids.

A recurring theme in defense allocation theories is the assumption that there is a trade-off between growth and defense (see Herms and Mattson 1992). This has led to numerous attempts to find a trade-off between plant fitness and the level of constitutive and induced defense (Haukioja *et al.* 1998, Koricheva *et al.* 1998, Warren *et al.* 1999). The CNB hypothesis predicts that fertilization will increase growth at the expense of C-based (*e.g.* phenolics and terpenoids) production of secondary metabolites (Bryant *et al.* 1983, Herms and Mattson 1992). In this study, the results support the CNB theory in induced defense reactions of N-fertilized trees, but not in unwounded trees and with other fertilizations. Estimation of the cost of the defense reaction

involves several unknown aspects; in many organisms the costs can be partly the costs for traits that are strongly correlated with defense but are not themselves defensive. The currency used, individual carbohydrates or total soluble carbohydrates, may not be representative either (**IV**). In mature trees, which have already passed the most active growth phase, it is difficult to relate minor changes in current growth rate to defense reactions. Difficulties in quantifying and detecting the costs of constitutive defense from induced defense are also relevant. The phloem probably represents the accumulation of photosynthates over a longer period of time, about 15–30 years of growth. Hamilton *et al.* (2001) have argued that CNB theory should be replaced with a new theory based on hypotheses that have an evolutionary underpinning that presupposed an adaptive value of any trait. In the long run we will see whether limitations in the predictive tool of CNB hypothesis will lead to the final formulation of the new theory that successfully predicts concentrations of defense-related secondary compounds.

The question of whether N fertilization decreases defense due to increased growth was partly answered. Vigour indices and diameter growth were higher in N-fertilized treatments than in the control and with P fertilization (**IV**). Diameter growth and the vigour index correlated positively with the length of the lesion caused by *C. polonica* inoculation. Because fertilization increased the diameter of the experimental trees, density of the resin duct may have decreased as a result of increased cell division. This may partly explain why the total terpene concentration of N-fertilized trees was lower than in the control (**III**). Interestingly, fertilization did not affect total concentration of soluble carbohydrates, although with fertilization the growth of experimental trees improved significantly (**IV**). Thus the differences in carbohydrate composition after wounding are supported by the histological observations of Nagy *et al.* (2000). When the two-month incubation time and the low density of the inoculations are taken into account, short lesions probably indicate successful defense with minimum loss of energy and resources. Overall, when tested on artificial media, many *Ceratocystis* species are able to utilise various nitrogen sources (Mathiesen-Käärik 1960). Accordingly in the present study, carbon resources probably were not the restricting factor in defense reactions.

3.3 Beetle-fungi-host tree interaction

Various morphological, chemical and behavioural modifications in bark beetles and the ophiostomatoid fungi ensure a close association. Spruce bark beetles are among the phloem-feeding insects that benefit from association with fungi mainly because fungi reduce tree resistance, thus increasing the breeding success of the beetles. Although the first beetles in an attack may be overwhelmed and killed by the secondary compounds of the host tree, they may inoculate the tree with fungi that may linger for a long time. The fungi are dependent on the bark beetles for transport and for inoculation into suitable habitats. The potential of the fungi to weaken and predispose trees to subsequent attacks has been considered important, especially when the density of the beetle population has been low and there have not been enough beetles to mount successful mass attacks (Berryman 1982).

As the vigour of Norway spruce increases, experimental trees tolerate an increasing number of spruce bark beetle attacks without being killed (Mulock and Christiansen 1986). According to Reid and Robb (1999), trees killed while growing vigorously, such as those felled by windthrow, may contribute significantly to initial increases in population, leading from endemic to epidemic populations of bark beetles. They suggest that the importance of phloem quality might be the key determinant of bark beetle performance. Bark beetle-fungal infection is only slightly inhibited by constitutive compounds; thus the induced response of the host is elicited and is essential in stopping invasion (Klepzig *et al.* 1996). In an elegant series of bioassays, Klepzig *et al.* (1996) showed that beetle responses to induced reactions could be a component of the host selection process. The beetles probably discriminate between susceptible host trees and vigorous trees capable of mounting an effective defense.

The above-mentioned ideas have been united by characterization of the important role of fungal infection in activating PP cells quickly throughout several cell layers and launching a systemic induced defence reaction far away from the original site of infection (Franceschi *et al.* 2000). Some recent results have also supported the idea that mechanical stress, rather than associated fungi, plays an essential role in induction and development of the response of trees to bark beetles (Lieutier *et al.* 1995). Mechanically made control wounds were here cleaned effectively and sealed with constitutive resin, whereas only fungal inoculations caused induced accumulation of secondary compounds (**III**). However, both mechanical wounding and fungal infection of Norway spruce have resulted in enhanced

resistance to subsequent mass inoculation with *C. polonica* (Christiansen *et al.* 1999).

One artificial inoculation cannot be considered to correspond to one bark beetle attack because artificial inoculations lead more surely to fungal infection of the phloem than to bark beetle attacks (Christiansen 1985b). The number of spores in one inoculum, 5 mm diameter slant of the colony of *Ophiostoma* fungus, can contain 1.5×10^6 spores (Lieutier *et al.* 1989). In natural infection, gallery construction by the beetles will spread the fungal spores more efficiently over a larger area than artificial point inoculations do. In addition, in natural bark beetle attacks, growth conditions are probably more favourable for fungi. Occasionally, the attacking beetles may not carry enough fungal spores for infection (Bridges and Moser 1983). Thus, the inoculation density might be more important than the fungal load per inoculation. An increase in the density of inoculations above a certain level has been reported to result in decreased resinosis (Raffa and Berryman 1983, Christiansen 1985a).

In low-density inoculations the ultimate reason for the long reaction zones may be the aggressiveness of fungus or the weakness of the host. Lesion length may vary depending on the season when trees are inoculated and on the length of the incubation period (Raffa and Smalley 1988, Parmeter *et al.* 1992). As used here, an incubation period of 8–10 weeks probably provides data from near the endpoint, *i.e.* when all defense reactions elicited by wounding are completed (Parmeter *et al.* 1992, **III**, **IV**). According to Solheim (1988), *C. polonica* produced slightly shorter lesions than the less aggressive *O. penicillatum* did. According to Popp *et al.* (1995), large lesions with a high monoterpene concentration in secondary resin also reflect greater fungal virulence rather than greater host resistance. Thus, lesion length alone may not be a good measure of the aggressiveness of fungi or the resistance of trees. Lesion length should be considered a supporting and descriptive parameter, not a main parameter judging solely the resistance (**III**, **IV**). With low inoculation level, a tree saves energy and allocates resources to defense by forming a reaction zone that is just large enough to prevent the growth of fungi. However, with low inoculation density, resin exudation is expected to be powerful, since the tree response is not exhausted immediately.

Different bark beetles can have specific fungal associates, which occur only with their host species (Jacobs and Wingfield 2001). Both ophiostomatoid fungi and spruce bark beetles can exist and develop without their associates, but in nature they are commonly found together; the relationship is nearly symbiotic.

C. polonica, *C. minuta*, *O. bicolor* and *O. penicillatum* were constant associates of *I. typographus* (**I, II**). *C. polonica* has been isolated mainly from spruce bark beetles (Solheim 1986, Kirisits 1996, Krokene and Solheim 1996, **I**, **II**) whereas the rest of the ophiostomatoid species mentioned here occur together with several bark beetles and some of them also spread by air. Species were classified here according to their frequency of occurrence (Table 2). Some fungi can be classified as constant associates of *I. typographus*, while others are common or casual associates.

Nevertheless, it is difficult to decide whether the symbiosis between beetles and specific fungi is obligate. The success of rearing sterile adult beetles alone does not rule out the presence of a symbiotic association. It is possible to rear generations of sterile bark beetles *in vitro* with species that normally are associated with fungi (Grosmann 1931, Harding 1989). Thus, the presence of fungi is not a prerequisite for larval nutrients and establishment of the spruce bark beetle, in spite of all the evidence pointing to a symbiotic relationship between the beetle and fungi. Also in natural conditions successful bark beetle infestations have been found without a constant fungal associate, like *Dendroctonus frontalis* Zimmermann infestations without *Ceratocystis minor* (Hedgcock) Hunt (Bridges *et al.* 1985).

In addition, different associated fungi can have a variable role in different interactions and under different environmental conditions (Harding 1989, Lieutier *et al.* 1995). According to Paine *et al.* (1988), mycangial fungi did not trigger the induced wound response in host trees compared with sterile wounding, whereas the less adapted or less specialized non-mycangial *C. minor* did induce lesion formation. The virulence of ophiostomatoid fungi may differ geographically, and the resistance of host trees may vary in different environments. Recent investigations (Stout *et al.* 1998) point out that induced resistance responses show variation in elicitation specificity and the organism involved. To understand the interaction between variable defensive reactions against stress factors and how physiological processes affect these responses, studies that take into account the specificity of induced responses are needed. Investigations are also needed to evaluate different strains of ophiostomatoid species; thus variation in sporulation ability and frequency (**I, II**) and virulence (Kirisits 1996, 1998) indicates variation within the current species concepts.

Table 2. Comparison of basic characteristics of *I. typographus* associated ophiostomatoid fungi. Intimacy of association refers the relationship between spruce bark beetle and associated fungi. Constant = fungus present > 90 % of studied beetles; common = fungus present 89–11 % of studied beetles; casual = fungus present < 10 % of studied beetles. Abbreviations of bark beetle names: *D.ruf* = *Dendroctonus rufipennis*, *D.val* = *Dendroctonus valens*, *Dryo* = *Dryocoetes* sp., *H.gla* = *Hylorgops glabratus*, *H.pal* = *Hylorgops palliatus*, *I.ami* = *Ips amitinus*, *I.cem* = *Ips cembrae*, *I.dup* = *Ips duplicatus*, *I.typo* = *I. typographus*, *P.cha* = *Pityogenes chalcographus*, *P.pol* = *Polygraphus poligraphus*, *T.pini* = *Tomicus piniperda*.

Species	Ascospore shape	Perithecium base size, µm	Perithecium neck legth, µm	Dispersal agent	Intimacy of association
C. minuta	orange section-shape, falcate	49-111 [2] 60-80 [9] 48-87 [11]	42-148 [2] 36-250 [9] 67-151 [11]	*I.ami* [3], *I.cem* [3], *I.typo* [1-3,5-7,9] *P.cha* [2,3], *T.pini* [3]	common [9] casual [1,2]
C. polonica	ellipsoid, cucullate	186-392 [2] 212-346 [8] 200-390 [11]	578-1049 [2] 356-978 [8] 360-1060 [9] 600-1150 [11]	*H.pal* [4], *I.ami* [3], *I.typo* [1-9] *I.dup* [4], *P.cha* [2-4], *P.pol* [4]	constant [4,6] common [5,7,9] casual [8]
O. ainoae	cylindrical	90-140 [5] 97-142 [8] 110-150 [9]	350-570 [5] 422-497 [8] 640-850 [9]	*Dryo* [3], *H.gla* [2,3], *H.pal* [3], *I.typo* [1-3,5-8], *P.cha* [3]	common [2,6] casual [5,8]
O. bicolor	cylindrical, oblong	206-333 [2] 245-322 [8] 310-340 [9]	274-1000 [2] 575-940 [8] 670-740 [9]	*I.ami* [3], *I.cem* [3], *I.dup* [4], *I.typo* [1-9], *P.cha* [2-4], *P.pol* [4]	common [3-9]
O. cucullatum	mandarin slice-shape	93-137 [2] 76-110 [5] 60-70 [9]	323-695 [2] 260-560 [5] 300-410 [9]	*Dryo* [3], *I.ami* [3], *I.typo* [1-3,5], *H.gla* [3], *H.pal* [3], *P.cha* [3]	casual [1,2,5]
O. flexuosum	cylidrical, ossiform	98-196 [2] 125-160 [5]	441-1029 [2] 650-940 [5]	*H.gla* [2,3], *I.typo* [1,5]	casual [1,5]
O. piceaperdum	hat-shape	186-303 [2] 250-327 [8] 200-310 [9] 180-360 [10]	196-686 [2] 169-282 [8] 180-900 [9] 256-1128 [10]	*D.ruf* [10], *D.val* [10], *Dryo* [3,10], *H.gla* [1-3], *H.pal* [1,3,4,10], *I.ami* [3], *I.dup* [4], *I.typo* [1-10], *P.cha* [2,3], *P.pol* [4], *T.pini* [3]	constant [1,2,8] casual [6,9]
O. penicillatum	allantoid, cylindrical	196-362 [2] 203-270 [8] 220-260 [9]	294-1186 [2] 192-712 [8] up to 1200 [9,11]	*I.ami* [3], *I.dup* [4], *I.typo* [1-9], *P.cha* [3], *P.pol* [4]	common [4,6,8,9]
O. piceae	orange section-shape	121-197 [2] 209-310 [8] 180-200 [9]	411-1009 [2] 475-1119 [8] up to 1530 [9]	*Dryo* [3], *H.gla* [2,3], *H.pal* [3,4] *I.ami* [3], *I.cem* [3], *I.dup* [4], *I.typo* [1-9], *P.cha* [3,4], *T.pini* [3]	casual [1,2,5-7,9] common [4,8]
O. tetropii	allantoid	373-475 [8]	435-706 [8]	*I.typo* [5,6-8]	casual [5,6,8]

References: 1=Harding 1989; 2-3=Kirisits 1996, 2000; 4=Krokene and Solheim 1996; 5-7=Solheim 1986, 1992b, 1993; 8=Viiri 1997; 9=Viiri and Lieutier, Paper II in this volume; 10=Wright and Cain 1961 and 11=Yamaoka *et al.* 1997.

Conclusions

The species complement of fungi associated with *I. typographus* was not affected by collecting method of beetles (**I**). However, the species composition of ophiostomatoid fungi varied according to the geographical region. The species considered to being most pathogenic, *C. polonica*, occurred with low frequency, regardless of the population level of spruce bark beetle (**I, II**). Benefits from the relationship for the bark beetles or disadvantages for the host trees are more complex. Trees responded extensively to fungal invasion (**III**), and the extent of this response was dependent on carbohydrate reserves (**IV**). Fertilization enhanced stem growth, but the total amount of soluble carbohydrates near the inoculation site was not affected. Resources for stem growth were not taken from defense (**IV**). Paine *et al.* (1997) have called the strategy of this interaction "exhausting tree resistance" rather than tree killing. On the base of the present results, extensive terpenoid accumulation in induced defense response seems more like exhausting tree resistance. However, interactions should always be viewed together with all partners, fungi - bark beetle and host tree.

References

Annila, E. 1969. Influence of temperature upon the development and voltinism of Ips typographus L. (Coleoptera, Scolytidae). Ann. Zool. Fenn. 6: 161-208.

— 1977. Seasonal flight patterns of spruce bark beetles. Ann. Ent. Fenn. 43: 31-35.

Austaråa, Ø., Annila, E., Bejer, B. & Ehnström, B. 1983. Insect pests in forests of the Nordic countries 1977-1981. Fauna Nor. Ser. B 31: 8 15.

Bakke, A. 1981. Inhibition of the response in Ips typographus to the aggregation pheromone; field evaluation of verbenone and ipsenol. Z. ang. Ent. 92: 172-177.

— 1983. Host tree and bark beetle interaction during a mass outbreak of Ips typographus in Norway. Z. ang. Ent. 96: 118-125.

— , Austaråa, Ø. & Pettersen, H. 1977a. Seasonal flight activity and attack pattern of Ips typographus in Norway under epidemic conditions. Medd. Nor. Inst. Skogforsk. 33: 253-268.

— , Frøyen, P. & Skattebøl, L. 1977b. Field response to a new pheromonal compound isolated from Ips typographus. Naturwissenschaften 64: 98.

Beaver, R.A. 1989. Insect-Fungus Relationships in the Bark and Ambrosia Beetles. In: Wilding, N., Collins, N.M., Hammond, P.M. & Webber, J.F. (eds.). Insect-Fungus Interactions. Academic Press, London. Pp. 121-143.

Berryman, A.A. 1969. Responses of Abies grandis to attack by Scolytus ventralis (Coleoptera: Scolytidae). Can. Entomol. 101: 1033-1041.

— 1972. Resistance of conifers to invasion by bark beetle-fungus associations. Bioscience 22: 598-602.

— 1982. Population Dynamics of Bark Beetles. In: Mitton, J. B. & Sturgeon, K. B. (eds.). Bark Beetles in North American Conifers. - A System for the Study of Evolutionary Biology. University of Texas Press, Austin. Pp. 264-314.

Birgersson, G. 1989. Host tree resistance influencing pheromone production in Ips typographus (Coleoptera: Scolytidae). Holarct. Ecol. 12: 451-456.

— , Schlyter, F., Löfqvist, J. & Bergström, G. 1984. Quantitative variation of pheromone components in the spruce bark beetle Ips typographus from different attack phases. J. Chem. Ecol. 10: 1029-1055.

Bordasch, R.P. & Berryman, A.A. 1977. Host resistance to the fir engraver beetle, Scolytus ventralis (Coleoptera: Scolytidae) 2. Repellency of Abies grandis resins and some monoterpenes. Can. Entomol. 109: 95-100.

Boutte, B. 1993. Le typographe de l'epicéa. In: La Santé des Forêts [France] en 1992. Département de la Santé des Forêts, Ministère de

l'agriculture, et de la pêche et de l'álimentation, Paris. Pp. 13-15. [In French].
Bridges, J.R. 1987. Effects of terpenoid compounds on growth of symbiotic fungi associated with the southern pine beetle. Phytopathology 77: 83-85.
— & Moser, J.C. 1983. Role of two phoretic mites in transmission of bluestain fungus, Ceratocystis minor. Ecol. Entomol. 8: 9-12.
— , Nettleton, W.A. & Connor, M.D. 1985. Southern pine beetle (Coleoptera: Scolytidae) infestations without the bluestain fungus, Ceratocystis minor. J. Econ. Entomol. 78: 325-327.
Brignolas, F., Lacroix, B., Lieutier, F., Sauvard, D., Drouet, A., Claudot, A.-C., Yart, A., Berryman, A.A. & Christiansen, E. 1995. Induced responses in phenolic metabolism in two Norway spruce clones after wounding and inoculations with Ophiostoma polonicum, a bark beetle-associated fungus. Plant Physiol. 109: 821-827.
— , Lieutier, F., Sauvard, D., Christiansen, E. & Berryman, A.A. 1998. Phenolic predictors for Norway spruce resistance to the bark beetle Ips typographus (Coleoptera: Scolytidae) and an associated fungus, Ceratocystis polonica. Can. J. For. Res. 28: 720-728.
Bryant, J.P., Chapin, F.S., III & Klein, D.R. 1983. Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. Oikos 40: 357-368.
Christiansen, E. 1985a. Ceratocystis polonica inoculated in Norway spruce: Blue-staining in relation to inoculum density, resinosis and tree growth. Eur. J. For. Pathol. 15: 160-167.
— 1985b. Ips/Ceratocystis-infection of Norway spruce: what is a deadly dosage? Z. ang. Ent. 99: 6-11.
— & Bakke, A. 1988. The Spruce Bark Beetle of Eurasia. In: Berryman, A. A. (ed.). Dynamics of Forest Insect Populations. Patterns, Causes, Implications. Plenum Press, New York. Pp. 479-503.
— & Ericsson, A. 1986. Starch reserves in Picea abies in relation to defence reaction against a bark beetle transmitted blue-stain fungus, Ceratocystis polonica. Can. J. For. Res. 16: 78-83.
— & Horntvedt, R. 1983. Combined Ips/Ceratocystis attack on Norway spruce, and defensive mechanisms of the trees. Z. ang. Ent. 96: 110-118.
— , Krokene, P., Berryman, A.A., Franceschi, V.R., Krekling, T., Lieutier, F., Lönneborg, A. & Solheim, H. 1999. Mechanical injury and fungal infection induce acquired resistance in Norway spruce. Tree Phys. 19: 399-403.
— , Waring, R.H. & Berryman, A.A. 1987. Resistance of conifers to bark beetle attack: Searching for general relationships. For. Ecol. Manage. 22: 89-106.
Cobb, F.W., Jr., Krstic, M., Zavarin, E. & Barber, H.W., Jr. 1968. Inhibitory effects of volatile oleoresin components on Fomes annosus and four Ceratocystis species. Phytopathology 58: 1327-1335.
Cook, S.P. & Hain, F.P. 1985. Qualitative examination of the hypersensitive response of loblolly pine, Pinus taeda L., inoculated with two fungal associates of the southern pine beetle, Dendroctonus frontalis

Zimmermann (Coleoptera: Scolytidae). Environ. Entomol. 14: 396-400.

— 1987. Four parameters of the wound response of loblolly and shortleaf pines to inoculation with the blue-staining fungus associated with the southern pine beetle. Can. J. Bot. 65: 2403-2409.

Davidson, R.W. 1953. Two common lumber-staining fungi in the western United States. Mycologia 45: 579-586.

— , Francke-Grosmann, H. & Käärik, A. 1967. A Restudy of Ceratocystis penicillata and report of two American species of this genus from Europe. Mycologia 59: 928-932.

— & Robinson-Jeffrey, R.C. 1965. New records of Ceratocystis europhioides and C. huntii with Verticicladiella imperfect stages from conifers. Mycologia 57: 488-490.

De Groot, R.C. 1972. Growth of wood-inhabiting fungi in saturated atmospheres of monoterpenoids. Mycologia 64: 863-870.

De Hoog, G.S. & Scheffer, R.J. 1984. Ceratocystis versus Ophiostoma: A Reappraisal. Mycologia 76: 292-299.

Delorme, L. & Lieutier, F. 1990. Monoterpene composition of the preformed and induced resins of Scots pine, and their effect on bark beetles and associated fungi. Eur. J. For. Pathol. 20: 304-316.

Dowding, P. 1969. The dispersal and survival of spores of fungi causing bluestain in pine. Trans. Br. Mycol. Soc. 52: 125-137.

Evensen, P.C., Solheim, H., Høiland, K. & Stenersen, J. 2000. Induced resistance of Norway spruce, variation of phenolic compounds and their effects on fungal pathogens. For. Path. 30: 97-108.

Franceschi, V.R., Krekling, T., Berryman, A.A., & Christiansen, E. 1998. Specialized phloem parenchyma cells in Norway spruce (Pinaceae) bark are an important site of defense reactions. Am. J. Bot. 85: 601-615.

— , Krokene, P., Krekling, T. & Christiansen, E. 2000. Phloem parenchyma cells are involved in local and distant defense responses to fungal inoculation or bark-beetle attack in Norway spruce (Pinaceae). Am. J. Bot. 87: 314-326.

Francke-Grosmann, H. 1963. Some new aspects in forest entomology. Annu. Rev. Entomol. 8: 415-438.

Furniss, M.M., Solheim, H. & Christiansen, E. 1990. Transmission of blue-stain fungi by Ips typographus (Coleoptera: Scolytidae) in Norway spruce. Ann. Entomol. Soc. Am. 83: 712-716.

Gershenzon, J. 1994. The Cost of Plant Chemical Defense Against Herbivory: a Biochemical Perspective. In: Insect-Plant Interactions, ed. E.A. Bernays. Volume V. CRC Press, Boca Raton. Pp. 105-173.

Grosmann, H. 1931. Beiträge zur Kenntnis der Lebensgemeinschaft zwischen Borkenkäfern und Pilzen. Z. für Parasitenkunde 3: 56-102. [In German].

Hamilton, J.G., Zangerl, A.R., DeLucia, E.H. & Berenbaum, M.R. 2001. The carbon-nutrient balance hypothesis: its rise and fall. Ecol. Lett. 4: 86-95.

Harding, S. 1989. The influence of mutualistic blue stain fungi on bark

beetle population dynamics. Ph.D. thesis, Department of Zoology, Royal Veterinary and Agricultural University. Copenhagen.
Harrington, T.C. 1981. Cycloheximide sensitivity as a taxonomic character in Ceratocystis. Mycologia 73: 1123-1129.
— 1987. New combinations in Ophiostoma and Ceratocystis species with Leptographium anamorphs. Mycotaxon 28: 39-43.
— , Steimel, J.P., Wingfield, M.J. & Kile, G.A. 1996. Isozyme variation and species delimitation in the Ceratocystis coerulescens complex. Mycologia 88: 104-113.
— , McNew, D., Steimel, J., Hofstra, D. & Farrell, R. 2001. Phylogeny and taxonomy of the Ophiostoma piceae complex and the Dutch elm disease fungi. Mycologia 93: 111-136.
Hart, J.H. 1981. Role of phytostilbenes in decay and disease resistance. Annu. Rev. Phytopathol. 19: 437-458.
Haukioja, E., Ossipov, V., Koricheva, J., Honkanen, T., Larsson, S. & Lempa, K. 1998. Biosynthetic origin of carbon-based secondary compounds: cause of variable responses of woody plants to fertilization? Chemoecology 8: 133-139.
Hausner, G., Reid, J. & Klassen, G.R. 2000. On the phylogeny of members of Ceratocystis s.s. and Ophiostoma that possess different anamorphic states, with emphasis on the anamorph genus Leptographium, based on partial ribosomal DNA sequences. Can. J. Bot. 78: 903-916.
Hawksworth, D.L., Kirk, P.M., Sutton, B.C. & Pegler, D.N. (eds.) 1995. Ainsworth & Bisby's Dictionary of the Fungi. 8. Edition. Commonwealth Mycological Institute. Kew, Surrey. 616 p.
Herms, D.A. & Mattson, W.J. 1992. The dilemma of plants: to grow or defend. Q. Rev. Biol. 67: 283-335.
Hodges, J.D., Elam, W.W., Watson, W.F. & Nebeker, T.E. 1979. Oleoresin characteristics and susceptibility of four southern pines to southern pine beetle (Coleoptera: Scolytidae) attacks. Can. Entomol. 111: 889-896.
Horntvedt, R., Christiansen, E., Solheim, H. & Wang, S. 1983. Artificial inoculation with Ips typographus-associated blue-stain fungi can kill healthy Norway spruce trees. Medd. Nor. Inst. Skogforsk. 38: 1-20.
Ivarsson, P. 1995. The pheromone systems of the spruce bark beetles, Ips duplicatus and I. typographus: biosynthesis and regulation. Dissertation, Chemical Ecology, Faculty of Natural Sciences, Göteborg University.
Jacobs, K., Wingfield, M.J. & Crous, P.W. 2000. Ophiostoma europhioides and Ceratocystis pseudoeurophioides, synonyms of O. piceaperdum. Mycol. Res. 104: 238-243.
— & van Wyk, P.W.J. 1996. Ultrastructural study of centrum development and ascospore morphology in Ophiostoma europhioides. S. Afr. J. Sci. 92: 386-390.
Jacobs, K. & Wingfield, M.J. 2001. Leptographium species. Tree Pathogens, Insect Associates, and Agents of Blue-Stain. APS Press, The American Phytopathological Society, St. Paul,

Minnesota. 207 p.
Kendrick, W.B. 1962. The Leptographium complex Verticicladiella Hughes. Can. J. Bot. 40: 629-770.
Kirisits, T. 1996. Investigations on the association of blue-stain fungi (Ophiostoma/Ceratocystis spp.) with the phloem-feeding spruce bark beetles Ips typographus L., Pityogenes chalcographus L. and Hylurgops glabratus Zett. in Austria. Master Thesis. Insitute of Forest Entomology, Forest Pathology and Forest Protection, Universität für Bodenkultur, Wien. 175 p. [In German]
— 1998. Pathogenicity of three blue-stain fungi associated with the bark beetle Ips typographus to Norway spruce in Austria. Österr. Z. Pilzk. 7: 191-201.
— & Anglberger, H. 1999. Report on a strain of the pathogenic blue-stain fungus Ceratocystis polonica with low virulence. Österr. Z. Pilzk. 8: 157-166.
— , Grubelnik, R. & Führer, E. 2000. Die ökologische Bedeutung von Bläuepilzen für rindenbrütende Borkenkäfer. In: Mariabrunner Waldbautage 1999 – Umbau sekundärer Nadelwälder. (ed.) F. Müller. FBVA-Berichte, Schriftenreihe der Forstlichen Bundesversuchsanstalt, Wien. Nr. 111. Pp. 117-137. [In German]
Klepzig, K.D., Smalley, E.B. & Raffa, K.F. 1996. Combined chemical defenses against an insect-fungal complex. J. Chem. Ecol. 22: 1367-1388.
Klimetzek, D. & Yue, C. 1997. Climate and forest insect outbreaks. Biológia 52: 153-157.
Koricheva, J., Larsson, S., Haukioja, E. & Keinänen, M. 1998. Regulation of woody plant secondary metabolism by resource availability: hypothesis testing by means of meta-analysis. Oikos 83: 212-226.
Krekling, T., Franceschi, V.R., Berryman, A.A. & Christiansen, E. 2000. The structure and development of polyphenolic parenchyma cells in Norway spruce (Picea abies) bark. Flora 195: 354-369.
Krokene, P. & Solheim, H. 1996. Fungal associates of five bark beetle species colonizing Norway spruce. Can. J. For. Res. 26: 2115-2122.
— 1998. Pathogenicity of four blue-stain fungi associated with aggressive and nonaggressive bark beetles. Phytopathology 88: 39-44.
— 2001. Loss of pathogenicity in the blue-stain fungus Ceratocystis polonica. Plant Pathology 50: 497-502.
Lanne, B.S., Ivarsson, P., Johnsson, P., Bergström, G. & Wassgren, A-B. 1989. Biosynthesis of 2-methyl-3-buten-2-ol, a pheromone component of Ips typographus (Coleoptera: Scolytidae). Insect Biochem. 19: 163-167.
Leufvén, A., Bergström, G. & Falsen, E. 1984. Interconversion of verbenols and verbenone by identified yeasts isolated from the spruce bark beetle Ips typographus. J. Chem. Ecol. 10: 1349-1361.
— , & Nehls, L. 1986. Quantification of different yeasts associated with the bark beetle, Ips typographus, during its attack on a spruce tree. Microb. Ecol. 12: 237-243.
Lieutier, F. & Berryman, A.A. 1988. Elicitation of Defensive Reactions in Conifers. In: Mattson, W.J., Levieux, J. & Bernard-Dagan, C.

(eds.). Mechanisms of Woody Plant Defenses Against Insects. Search for Pattern. Springer-Verlag, New York. Pp. 313-319.
— , Cheniclet, C. & Garcia, J. 1989. Comparison of the defense reactions of Pinus pinaster and Pinus sylvestris to attacks by two bark beetles (Coleoptera: Scolytidae) and their associated fungi. Environ. Entomol. 18: 228-234.
— , Garcia, J., Yart, A. & Romary, P. 1995. Wound reactions of Scots pine (Pinus sylvestris L.) to attacks by Tomicus piniperda L. and Ips sexdentatus Boern. (Col., Scolytidae). J. Appl. Entomol. 119: 591-600.
Lindberg, M., Lundgren, L., Gref, R. & Johansson, M. 1992. Stilbenes and resin acids in relation to the penetration of Heterobasidion annosum through the bark of Picea abies. Eur. J. For. Pathol. 22: 95-106.
Lindgren, B.S., Nordlander, G. & Birgersson, G. 1996. Feeding deterrence of verbenone to the pine weevil, Hylobius abietis (L.) (Col., Scolytidae). J. Appl. Entomol. 120: 397-403.
Löyttyniemi, K., Austarå, Ø., Bejer, B. & Ehnström, B. 1979. Insect pests in forests of the Nordic countries 1972-1976. Folia For. 395: 1-13.
— & Uusvaara, O. 1977. Insect attack on pine and spruce sawlogs felled during the growing season. Commun. Inst. For. Fenn. 89: 1-48.
Mannila, E. 1993. Biologically active stilbene derivatives from Picea abies bark by isolation and modification. Annales Academiæ Scientiarum Fennicæ, Series A, II. Chemica, 245. Dissertation, Department of Chemistry, University of Jyväskylä. 40 p. + 5 reprints.
Mathiesen A. 1951. Einige neue Ophiostoma-arten in Schweden. Svensk Bot. Tidskr. 45: 203-232. [In German with English summary].
Mathiesen-Käärik, A. 1953. Eine Übersicht über die gewöhnlichsten mit Borkenkäfern assoziierten Bläuepilze in Schweden und einige für Schweden neue Bläuepilze. Medd. Statens skogsforskningsinst. 43: 1-74. [In German].
— 1960. Studies on the ecology, taxonomy and physiology of Swedish insect-associated blue stain fungi, especially the genus Ceratocystis. Oikos 11: 1-25.
Miller, R.H., Berryman, A.A. & Ryan, C.A. 1986. Biotic elicitors of defense reactions in lodgepole pine. Phytochemistry 25: 611-612.
Moser, J.C., Perry, T.J. & Solheim, H. 1989. Ascospores hyperphoretic on mites associated with Ips typographus. Mycol. Res. 93: 513-517.
Mulock, P. & Christiansen, E. 1986. The threshold of successful attack by Ips typographus on Picea abies: A field experiment. For. Ecol. Manage. 14: 125-132.
Münster-Swendsen, M. 1987. Index of vigour in Norway spruce (Picea abies Karst.). J. Appl. Ecol. 24: 551-561.
Nagy, N.E., Franceschi, V.R., Solheim, H., Krekling, T. & Christiansen, E. 2000. Wound-induced traumatic resin duct development in stems of Norway spruce (Pinaceae): Anatomy and cytochemical traits. Am. J. Bot. 87: 302-313.
Okada, G., Seifert, K.A., Takematsu, A., Yamaoka, Y., Miyazaki, S. & Tubaki, K. 1998. A molecular phylogenetic reappraisal of the

Graphium complex based on 18S rDNA sequences. Can. J. Bot. 76: 1495-1506.

— , Jacobs, K., Kirisits, T., Louis-Seize, G.W., Seifert, K.A., Sugita, T., Takematsu, A. & Wingfield, M.J. 2000. Epitypification of Graphium penicillioides Corda, with comments on the phylogeny and taxonomy of graphium-like synnematous fungi. Studies in Mycol. 45: 169-188.

Paine, T.D., Stephen, F.M. & Cates, R.G. 1988. Phenology of an induced response in loblolly pine following inoculation of fungi associated with the southern pine beetle. Can. J. For. Res. 18: 1556-1562.

— , Raffa, K.F. & Harrington, T.C. 1997. Interactions among scolytid bark beetles, their associated fungi, and live host conifers. Annu. Rev. Entomol. 42: 179-206.

Parmeter, J.R. Jr., Slaughter, G.W., Chen, M. & Wood, D.L. 1992. Rate and depth of sapwood occlusion following inoculation of pines with bluestain fungi. For. Sci. 38: 34-44.

Paulin-Mahady, A.E., Harrington, T.C. & McNew, D. 2002. Phylogenetic and taxonomic evaluation of Chalara, Chalaropsis, and Thielaviopsis anamorphs associated with Ceratocystis. Mycologia 94: 62-72.

Pohjola, J. 1993. A Headspace gas chromatographic study on the variation of needle volatile terpenes in Scots pine (Pinus sylvestris L.). Dissertation, Pharmacognosy Division, Department of Pharmacy, University of Helsinki. 177 p.

Popp, M.P., Johnson, J.D. & Lesney, M.S. 1995. Characterization of the induced response of slash pine to inoculation with bark beetle vectored fungi. Tree Phys. 15: 619-623.

Raffa, K.F. & Berryman, A.A. 1982. Accumulation of monoterpenes and associated volatiles following inoculation of grand fir with a fungus transmitted by the fir engraver, Scolytus ventralis (Coleoptera: Scolytidae). Can. Entomol. 114: 797-810.

— & Berryman, A.A. 1983. Physiological aspects of lodgepole pine wound responses to a fungal symbiont of the mountain pine beetle, Dendroctonus ponderosae (Coleoptera: Scolytidae). Can. Entomol. 115: 723-734.

— , Berryman, A.A., Simasko, J., Teal, W. & Wong, B.L. 1985. Effects of grand fir monoterpenes on the fir engraver, Scolytus ventralis (Coleoptera: Scolytidae), and its symbiotic fungus. Environ. Entomol. 14: 552-556.

— & Smalley, E.B. 1988. Seasonal and long-term responses of host trees to microbial associates of the pine engraver, Ips pini. Can. J. For. Res. 18: 1624-1634.

— & Smalley, E.B. 1995. Interaction of pre-attack and induced monoterpene concentrations in host conifer defense against bark beetle-fungal complexes. Oecologia 102: 285-295.

Ravn, H.P. 1985. Expansion of the populations of Ips typographus (L.) (Coleoptera, Scolytidae) and their local dispersal following gale disaster in Denmark. Z. ang. Ent. 99: 26-33.

Reid, M.L. & Robb, T. 1999. Death of vigorous trees benefits bark beetles. Oecologia 120: 555-562.

Reid, R.W., Whitney, H.S. & Watson, J.A. 1967. Reactions of lodgepole pine to attack by Dendroctonus ponderosae Hopkins and blue stain fungi. Can. J. Bot. 45: 1115-1126.

Risberg, B. 1985. Uppskattning av granbarkborreskador i Värmland 1971-1982 genom korrigering och komplettering av tidigare flyginventeringsresultat. Sveriges skogsvårdsförbunds tidskrift 2: 21-31. [In Swedish].

Rumbold, C.T. 1931. Two blue-staining fungi associated with bark-beetle infestation of pines. J. Agric. Res. 43: 847-873.

Russell, C.E. & Berryman, A.A. 1976. Host resistance to the fir engraver beetle. 1. Monoterpene composition of Abies grandis pitch blisters and fungus-infected wounds. Can. J. Bot. 54: 14-18.

Saalas, U. 1919. Kaarnakuoriaisista ja niiden aiheuttamista vahingoista Suomen metsissä. Valtioneuvoston kirjapaino, Helsinki. 374 p. [In Finnish].

— 1949. Suomen metsähyönteiset sekä muut metsälle vahingolliset ja hyödylliset eläimet. Wsoy, Porvoo. 719 p. [In Finnish].

Savonmäki, S. 1990. Tärkeimmät kaarnakuoriaisten mäntyyn ja kuuseen levittämät sinistäjäsienilajit. Master Thesis in Forest Pathology, Department of Plant Pathology, University of Helsinki. 43 p + 2 appendix. [In Finnish].

Schlyter, F. & Birgersson, G. 1989. Individual variation in bark beetle and moth pheromones – a comparison and an evolutionary background. Holarct. Ecol. 12: 457-465.

— , Löfqvist, J. & Byers, J.A. 1987. Behavioural sequence in the attraction of the bark beetle Ips typographus to pheromone sources. Physiol. Entomol. 12: 185-196.

Schowalter, T.D. & Filip, G.M. 1993. Beetle-Pathogen Interactions in Conifer Forests. Academic Press, London. 252 p.

Shrimpton, D.M. & Whitney, H.S. 1968. Inhibition of growth of blue stain fungi by wood extractives. Can. J. Bot. 46: 757-761.

Siemaszko, W. 1939. Zespoly grzybów towarzyszacych kornikom Polskim. Planta Polonica 7: 1-52. [Fungi associated with bark-beetles in Poland, in Polish with English summary]. ,c

Siitonen, J. 1998. Lahopuun merkitys metsäluonnon monimuotoisuudelle – kirjallisuuskatsaus. Finnish Forest Research Institute, Research Papers 705: 131-161. [In Finnish].

Solheim, H. 1986. Species of Ophiostomataceae isolated from Picea abies infested by the bark beetle Ips typographus. Nord. J. Bot. 6: 199-207.

— 1988. Pathogenicity of some Ips typographus-associated blue-stain fungi to Norway spruce. Medd. Nor. Inst. Skogforsk. 40: 1-11.

— 1991. Oxygen deficiency and spruce resin inhibition of growth of fungi associated with Ips typographus. Mycol. Res. 95: 1387-1392.

— 1992a. The early stages of fungal invasion in Norway spruce infested by the bark beetle Ips typographus. Can. J. Bot. 70: 1-5.

— 1992b. Fungal succession in sapwood of Norway spruce infested by the bark beetle Ips typographus. Eur. J. For. Pathol. 22: 136-148.

— 1993. Fungi associated with the spruce bark beetle Ips typographus in

an endemic area in Norway. Scand. J. For. Res. 8: 118-122.
— & Krokene. 1998. Growth and virulence of Ceratocystis rufipenni and three blue-stain fungi isolated from the Douglas-fir beetle. Can. J. Bot. 76: 1763-1769.
Stout, M.J., Workman, K.V., Bostock, R.M. & Duffey, S.S. 1998. Specificity of induced resistance in the tomato, Lycopersicon esculentum. Oecologia 113: 74-81.
Toscano Underwood, C.D. and Pearce, R.B. 1991. Variation in the levels of the antifungal stilbene glucosides astringin and isorhapontin in the bark of Sitka spruce [Picea sitchensis (Bong.) Carr.]. Eur. J. For. Pathol. 21: 279-289.
Upadhyay, H.P. & Kendrick, W.B. 1975. Prodromus for a revision of Ceratocystis (Microascales, Ascomycetes) and its conidial states. Mycologia 67: 798-805.
— 1981. A Monograph of Ceratocystis and Ceratocystiopsis. The University of Georgia Press. Athens, Georgia. 176 p.
Valkama, H., Räty, M. & Niemelä, P. 1997. Catches of Ips duplicatus and other non-target Coleoptera by Ips typographus pheromone trapping. Entomol. Fenn. 8: 153-159.
Van Wyk, P.W.J. & Wingfield, M.J. 1990. Ascospore development in Ceratocystis sensu lato (Fungi): a review. Bothalia 20: 141-145.
— 1993. Fine structure of ascosporogenesis in Ceratocystiopsis proteae. Can. J. Bot. 71: 1212-1218.
Viljoen, C.D., Wingfield, B.D. & Wingfield, M.J. 2000. Computer aided systematic evaluation of morphological characters of the ophiostomatoid fungi. Mycotaxon 74: 217-239.
Visser, C., Wingfield, M.J., Wingfield, B.D. & Yamaoka, Y. 1995. Ophiostoma polonicum is a species of Ceratocystis sensu stricto. Syst. Appl. Microbiol. 18: 403-409.
Wainhouse, D., Ashburner, R., Ward, E. & Boswell, R. 1998. The effect of lignin and bark wounding on susceptibility of spruce trees to Dendroctonus micans. J. Chem. Ecol. 24: 1551-1561.
Waring, R.H. & Pitman, G.B. 1983. Physiological stress in lodgepole pine as a precursor for Mountain pine beetle attack. Z. ang. Ent. 96: 265-270.
— , Thies, W.G. & Muscato, D. 1980. Stem growth per unit of leaf area: A measure of tree vigor. For. Sci. 26: 112-117.
Warren, J.M., Allen, H.L. & Booker, F.L. 1999. Mineral nutrition, resin flow and phloem phytochemistry in loblolly pine. Tree Phys. 19: 655-663.
Weijman, A.C.M. & De Hoog, G.S. 1975. On the subdivision of the genus Ceratocystis. Antonie Leeuwenhoek 41: 353-360.
Weslien, J., Annila, E., Bakke, A., Bejer, B., Eidmann, H.H., Narvestad, K., Nikula, A. & Ravn, H.P. 1989. Estimating risks for spruce bark beetle (Ips typographus (L.)) damage using pheromone-baited traps and trees. Scand. J. For. Res. 4: 87-98.
Whitney, H.S. 1982. Relationships between Bark Beetles and Symbiotic Organisms. In: Mitton, J.B. and Sturgeon, K.B. (eds.). Bark Beetles in North American Conifers. A System for the Study of Evolutionary

Biology. University of Texas Press, Austin. Pp. 183-211.
— & Blauel, R.A. 1972. Ascospore dispersion in Ceratocystis spp. and Europhium clavigerum in conifer resin. Mycologia 64: 410-414.
Wingfield, M.J. 1985. Reclassification of Verticicladiella based on conidial development. Trans. Br. Mycol. Soc. 85: 81-93.
— , Seifert, K.A. & Webber, J.F. 1993. Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity. APS Press, The American Phytopathological Society, St. Paul, Minnesota. 293 p.
— , De Beer, C., Visser, C. & Wingfield, B.D. 1996. A New Ceratocystis species defined using morphological and ribosomal DNA sequence comparisons. Syst. Appl. Microbiol. 19: 191-202.
Wong, B.L. & Berryman, A.A. 1977. Host resistance to the fir engraver beetle. 3. Lesion development and containment of infection by resistant Abies grandis inoculated with Trichosporium symbioticum. Can. J. Bot. 55: 2358-2365.
Woodward, S. & Pearce, R.B. 1988. The role of stilbenes in resistance of Sitka spruce (Picea sitchensis (Bong.) Carr.) to entry of fungal pathogens. Physiol. Mol. Plant Pathol. 33: 127-149.
Worrell, R. 1983. Damage by the spruce bark beetle in South Norway 1970-80: A survey, and factors affecting its occurrence. Medd. Nor. Inst. Skogforsk. 38: 1-34.
Wright, E. 1933. A cork-borer method for inoculating trees. Phytopathology 23: 487-488.
Wright, E.F. & Cain, R.F. 1961. New species of the genus Ceratocystis. Can. J. Bot. 39: 1215-1230.
Wright, L.C., Berryman, A.A. & Gurusiddaiah, S. 1979. Host resistance to the fir engraver beetle, Scolytus ventralis (Coleoptera: Scolytidae). 4. Effect of defoliation on wound monoterpene and inner bark carbohydrate concentrations. Can. Entomol. 111: 1255-1262.
Yamaoka, Y., Wingfield, M.J., Takahashi, I. & Solheim, H. 1997. Ophiostomatoid fungi associated with the spruce bark beetle Ips typographus f. japonicus in Japan. Mycol. Res. 101: 1215-1227.

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Fungal associates of the spruce bark beetle *Ips typographus* L. (Col. Scolytidae) in relation to different trapping methods

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Abstract: *Ips typographus* transports spores of various sapwood-staining fungi, mainly in the pits of the pronota and elytra. When pheromone trapping is used to collect beetles, the spores of fungi from different beetles may mix. Therefore, the species composition of fungi associated with *I. typographus* was investigated in eastern Finland using beetles caught by different trapping methods. The beetles were collected individually by hand or in pheromone traps with or without vermiculite used as insulating material in trap containers to prevent surface contamination between beetles. The beetles were inoculated into Norway spruce logs with a cork borer and the frequencies of fungi were determined by isolation of fungi from bark and sapwood. The most frequent ophiostomatoid species were *O. penicillatum* and *O. europhioides*. Other common fungi were *Graphium* and *Leptographium* species. The species that were isolated occasionally were *O. ainoae*, *O. bicolor*, *O. piceae*, *O. tetropii* and *C. polonica*. Relationships between fungal observations were analysed one by one. In pairwise comparison of pheromone trapping and individually collected beetles, the frequencies of fungi isolated from beetles differed significantly. However, when all trapping methods were compared simultaneously, the differences were not significant.

1 Introduction

Many species of fungi have been reported to occur in association with Scolytidae, the most numerous group belonging to the genera *Ophiostoma* H. and P. Sydow, *Ceratocystis* Ellis and Halsted, *Ceratocystiopsis* Upad. and Kendrick and related anamorph genera such as *Graphium* Corda and *Leptographium* Lagerb. and Melin (Mathiesen-Käärik, 1960; Upadhyay, 1981; Whitney, 1982; Solheim, 1986; Harrington, 1993). This economically important but taxonomically controversial group of fungi has recently been named the 'ophiostomatoid fungi' (Upadhyay, 1993).

While constructing breeding chambers and galleries in the phloem, beetles disseminate fungal spores. Many species of beetles have special organs, mycangia, in which spores are transported; and in some species slimy secretions are produced that preserve spores from desiccation and UV-light (Mathiesen-Käärik, 1960; Francke-Grosmann, 1963; Dowding, 1969). Ophiostomatoid fungi are adapted for dispersal by insects: elongated ascocarps bear mucilaginous ascospores in their necks, which may be further protected by gelatinous sheaths (Malloch and Blackwell, 1993); and the cirri of some *Ophiostoma* and *Ceratocystis* species disperse, not in water, but in conifer resin (Whitney and Blauel, 1972).

The Eurasian spruce bark beetle, *Ips typographus* L., transports spores of fungi laterally on the posterior half of the pronota, in pits throughout most of the elytra and in the digestive tract (Furniss et al., 1990). Spores have also been observed on phoretic mites associated with beetles (Moser et al., 1989). In *I. typographus* no slimy excretions have been described in the pits, where spores occur; but the spores may be covered with wax (E. Christiansen and H. Solheim, pers. comm.). Thousands of ascospores and conidia may be found on the surface of each beetle, but individuals vary greatly as to the number of spores transported (Furniss et al., 1990). Neither application of ultrasonic cleaning nor chemical sterilizing agents have completely eliminated fungal spores from adult *I. typographus* beetles. On the other hand, in spite of all the evidence pointing to a mutualistic relationship between *I. typographus* and ophiostomatoid fungi, the presence of fungi is not the prerequisite for establishment and successful reproduction of *I. typographus* (Harding, 1989c).

In several investigations *Ophiostoma bicolor* Davidson and Wells, *O. penicillatum* (Grosm.) Siem., *O. europhioides* (Wright and Cain) Solheim, and *C. polonica* (Siem.) Moreau have been associates of *I. typographus*. *C. polonica*, which is consistently associated with *I. typographus*, is pathogenic and can kill healthy Norway spruce trees (*Picea abies* L. Karst.) when mass inoculated into trees (Horntvedt et al., 1983; Christiansen, 1985). In addition, *O. europhioides* is able to invade the sapwood of Norway spruce and disrupt the water-conducting system (Harding, 1989b). *C. polonica* and *O. europhioides*, in particular, may play a special role in the population dynamics of the beetle. Frequencies of associated fungi are potential explanations for both changes in the beetle population and forest damage (Harding, 1989a,b; Solheim, 1993; Krokene and Solheim, 1996). Nonetheless, fungal frequencies are not independent observations if beetles have been in close contact in the collecting containers of traps and fungal spores may have mixed.

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This study was designed to examine how methods of trapping beetles affect on species of fungi found on *I. typographus*. The aim was to determine whether beetles collected (i) individually (ii) with pheromone traps using vermiculite as an insulating material in the collecting bottles and (iii) in pheromone traps without insulating material differ in frequency and abundance of fungi.

2 Materials and methods

2.1 Beetle collection

I. typographus beetles were collected in Liperi (N 62°31′; E 29°17′), eastern Finland, during the flight period (23 May–2 June 1992) on a forest area that had been clearcut the previous winter. The surrounding stand was Myrtillus-type with mature Norway spruce. Most of the beetles (60 individuals) were caught with a drain-pipe trap (model, 1979) without a funnel (BAKKE et al., 1983) with commercially prepared IPSLURE® plastic bag dispensers containing 1500 mg methylbutenol, 70 mg *cis*-verbenol and 15 mg ipsdienol. In each group of traps, four traps were placed in a 2 m × 2 m square. The three groups of traps (Liperi 1, Liperi 2 and Liperi 3) were located 50 m from the edge of the forest and 150 m from each other.

Every second day the traps were emptied and the container bottles sterilized with 70% alcohol. Before each collection period the containers in trap group Liperi 2 were filled with moist sterile vermiculite. Vermiculite was used in the trap containers to prevent possible mixing of fungal spores. Trap groups Liperi 1 and Liperi 3 were identical, and the containers remained empty without insulating material. Twenty beetles from each trap group were chosen as sample beetles. In addition to pheromone trapping, 20 beetles were collected individually by hand with sterile forceps from the outer surfaces of the traps and from adjacent spruce logs at the forest edge. Of the beetles caught, an equal number of females and males were selected. Until used in inoculations, they were stored individually at +4°C in sterile Eppendorf-test tubes containing a strip of filter paper moistened with sterile water.

2.2 Inoculations and isolations

Sample beetles, most of which were alive, were inoculated into 1 m long logs (15 cm diameter) cut from freshly felled, uninfected Norway spruce according to the method of FURNISS et al. (1990). The logs were brushed gently and washed with 0.5% 8-hydroxyquinoline sulphate–70% alcohol before the beetles were inserted. The unwashed beetles were placed 20 per log in a spiral pattern. Twenty control inoculations without beetles were made in a similar pattern. To prevent evaporation, the ends of the logs were dipped in melted paraffin.

After 4 weeks of incubation at room temperature, fungi were isolated from the inner bark and sapwood. The reaction lesion in the phloem usually consisted of an inner dark area and an outer lighter area, which was longer than wide. Within the reaction lesion, three samples were taken from both inner bark and sapwood. One sample was taken at the top of the light area of the lesion, one near the point of inoculation and one in the middle of the lesion area, at the centre of the previous samples. A total of 480 tissue samples were isolated and subcultured on 2% malt 1.5% agar to which 0.02% streptomycin had been added.

2.3 Statistical analysis

Frequencies of ophiostomatoid species from bark and sapwood samples were analysed either separately or combined, depending on the hypothesis tested. The combined frequencies were formed from the frequencies of bark and sapwood samples by counting the occurrence of a given species only once from one beetle. With data sets that were too large for exact calculation of P-value, the Monte-Carlo estimate of the P-value was formed by generating 100 000 tables. The level of significance applied in tests and Monte-Carlo estimates was $P < 0.05$. The data were analysed by StatXact™ Version 2.11 software (MEHTA and PATEL, 1989, 1991).

3 Results

3.1 Fungal composition

The most frequent ophiostomatoid species in both sapwood and bark were *O. penicillatum* and *O. europhioides*. Other common fungi were *Graphium* and *Leptographium* species (table 1). *O. ainoae* H. Solheim, *O. bicolor*, *O. piceae* (Münch) H. and P. Sydow, *O. tetropii* Mathiesen and *C. polonica* were isolated occasionally. Among the species other than ophiostomatoid fungi, *Nectria* spp. and *Bjerkandera adusta* (Willd. ex. Fr.) Karst. were common in isolations from both beetles and controls. *Penicillium* spp., light and dark sterile mycelia and several unknown species were isolated from control inoculations. However, no ophiostomatoid species were isolated from control inoculations.

3.2 Total number of fungal species

One to three species were isolated from 93% of the sapwood samples, and no more than five species were ever present on any one beetle (table 2). The Kruskal–Wallis test for sapwood samples indicated that the number of fungal species collected by different methods did not differ ($\chi^2 = 1.160$, DF 3, asymptotic P-value = 0.7625). 85% of the bark samples contained one to three species and the most species isolated from one beetle was five (table 2). The test for bark samples also supported the previous result that there was no variation in the number of species ($\chi^2 = 2.315$, DF 3, asymptotic P-value = 0.5097).

3.3 Occurrence in bark and sapwood

To test whether individual ophiostomatoid species were more likely to occur in bark or sapwood, contingency tables were used. An analysis series of 2 × 2 tables consisted of calculation of the odds ratios for all tables and a homogeneity test that the odds ratios are the same for all tables (table 3). In the homogeneity test ($\chi^2 < 0.0001$, DF 6) the Zelen exact P-value was 0.3867. The Monte-Carlo estimate of P-value gave a similar result. The hypothesis, i.e. that seven contingency tables formed from the occurrences of ophiostomatoid species in bark and sapwood samples share a common odds ratio, was accepted. For estimation of the common odds ratio, the Mantel–Haenszel method was used, which gave a value of 0.9356.

3.4 Associations among the fungi

A homogeneity test was used to test the hypothesis that some species are more likely than others to occur together. All possible pairs formed from the seven

Table 1. *Frequencies of fungi in bark (B) and sapwood (S) samples isolated from Norway spruce logs inoculated with* I. typographus *collected in Liperi, eastern Finland. Beetles were collected in pheromone traps with (Liperi 2) or without vermiculite (Liperi 1; Liperi 3) in trap containers or individually by hand using sterile forceps (individually trapped). (C = combined frequencies; n = 20 beetles in each trapping method)*

Trapping method	Liperi 1			Liperi 2			Liperi 3			Individually trapped			Total		
Isolation point	B	S	C	B	S	C	B	S	C	B	S	C	B	S	C
C. polonica	0	0	0	1	1	1	0	0	0	0	1	1	1	2	2
O. ainoae	0	0	0	0	0	0	0	0	0	1	3	3	1	3	3
O. bicolor	1	0	1	2	0	2	0	1	1	3	1	4	6	2	8
O. europhioides	3	4	6	3	1	3	3	1	4	1	1	2	10	7	15
O. penicillatum	6	4	8	2	2	4	4	4	7	1	1	2	13	11	21
O. piceae	0	2	2	0	2	2	1	1	2	1	1	2	2	6	8
O. tetropii	1	0	1	0	0	0	1	0	1	0	1	1	2	1	3
Graphium spp.	9	9	9	5	6	10	8	8	13	8	9	11	30	32	43
Leptographium spp.	9	5	11	6	2	6	5	3	6	8	5	10	28	15	33
Nectria spp.	6	4	7	2	2	3	7	3	8	4	1	4	19	10	22
B. adusta	2	1	3	2	0	2	2	1	3	4	4	8	10	6	16
Light sterile mycelia	8	7	12	8	6	10	10	6	12	5	7	8	31	26	42
Dark sterile mycelia	2	1	2	1	6	6	3	3	6	5	4	8	11	14	22
Unknown	4	8	8	7	9	11	1	5	2	7	6	10	19	28	31
No. of isolations	51	45	70	39	37	60	45	36	65	48	45	74	183	163	269

Table 2. *The total number of fungal species and groups isolated from* I. typographus *beetles inoculated in Norway spruce logs. Isolation points are B = Bark and S = Sapwood*

Amount of species	1 species		2 species		3 species		4 species		5 species		Number of
Isolation point	B	S	B	S	B	S	B	S	B	S	beetles
Liperi 1	7	6	3	7	6	5	4	2	0	0	20
Liperi 2	5	7	11	9	4	4	0	0	0	0	20
Liperi 3	5	7	5	8	6	5	4	0	0	0	20
Individually trapped	5	8	7	4	4	4	3	3	1	1	20
No. of fungal species	22	28	26	28	20	18	11	5	1	1	80

Table 3. *Empirical odds ratios (OR) with their 95% confidence intervals (CI) for occurrence of ophiostomatoid species in bark and sapwood of Norway spruce after inoculation with* I. typographus

Species	OR	95% CI
C. polonica	2.026	1.71, 3.13
O. ainoae	3.078	1.16, 3.41
O. bicolor	0.3162	0.48, 2.78
O. europhioides	0.6712	0.62, 1.42
O. penicillatum	0.8216	0.67, 1.07
O. piceae	3.162	0.48, 2.78
O. tetropii	0.4937	1.71, 3.13

species of ophiostomatoid fungi were included in the test. The Breslow–Day asymptotic P-value <0.001 ($\chi^2 = 110.8$, DF 20) was significant at the 0.1% level and the null hypothesis, that there is a common odds ratio across 21 pairs of fungal species, was rejected.

3.5 Comparison of frequencies of fungi with different trapping methods

Based on the previous analysis, combined frequencies of bark and sapwood samples were used in the Kruskal–Wallis analysis of variance to test the effect of trapping method on frequencies of fungi. When the individually collected beetles and those caught in traps were compared simultaneously, the differences were not significant (table 4). Furthermore, when trapping methods were compared and one trap group or the individually collected beetles were omitted, one at the time, from the comparison, none of the P-values were significant. In pairwise comparison of trapping methods, however, the differences were significant.

4 Discussion

4.1 Abundance of fungi

All ophiostomatoid fungi found in this study have been identified elsewhere as associates of *I. typographus* (Siemaszko, 1939; Harding, 1989a; Solheim, 1986, 1992a,b, 1993; Krokene and Solheim, 1996). Almost all occurred at lower frequencies than in non-epidemic areas of *I. typographus* in Denmark and Norway (Harding, 1989a; Solheim, 1993). A common species in this study, *O. penicillatum*, is also common in other Nordic countries (Harding, 1989a; Solheim, 1993; Krokene and Solheim, 1996). The relatively frequent occurrence of *O. europhioides* is in agreement only with the results

Table 4. *Frequencies of ophiostomatoid species isolated from spruce bark beetles caught with different trapping methods compared by Kruskal–Wallis analysis of variance. Trapping methods are: L1 = Liperi 1, L2 = Liperi 2, L3 = Liperi 3, I = Individually trapped)*

Trapping methods	Test value	Asymptotic P-value	DF
L1, L2, L3, I	5.593	0.1332	3
L1, L2, I	3.954	0.1385	2
L1, L3, I	5.397	0.0673	2
L2, L3, I	4.143	0.1260	2
L1, L2, L3	1.199	0.5490	2
L1, L2	0.7136	0.3982	1
L1, L3	0.0954	0.7574	1
L1, I	3.928	0.0475*	1
L2, L3	1.045	0.3067	1
L2, I	0.9353	0.3335	1
L3, I	3.968	0.0464*	1

Frequencies significantly different* = $P < 0.05$.

of Danish investigation (HARDING, 1989a) but differs from Norwegian results where *O. europhioides* has been found to be rare (SOLHEIM, 1986, 1993). The low frequency of pathogenic *C. polonica* associated with *I. typographus* in the present study is consistent with the low level of damage by beetles in eastern Finland. In Norway the frequency of *C. polonica* has been low during endemic periods when beetles utilize dead trees and timber, whereas the frequency has been higher during the epidemic phase when living trees are attacked (SOLHEIM, 1992a, 1993).

When the number of fungal species was investigated, neither asymptotic P-values for bark nor sapwood samples were significant at the 5% level; nevertheless, all Monte-Carlo estimates of P-values were close to the asymptotic P-values (not shown). These observations indicate that the asymptotic theory still worked despite the obvious sparseness of the data. According to these results, the total number of fungal species did not vary within trapping methods, thus allowing further analysis.

4.2 Occurrence in bark and sapwood

Odds ratios of less than one suggest that the odds of the fungi being successful are less in sapwood than in bark samples, while values greater than one indicate that the odds of success are greater in sapwood. The odds ratios for *O. bicolor*, *O. piceae* and *O. tetropii* were not within the 95% confidence intervals, but other odds ratios were within fairly narrow limits. The most abundant species, *O. europhioides* and *O. penicillatum*, seemed to colonize sapwood rather than bark. Due to the low frequency of *C. polonica,* the adaptation of species to invade the sapwood cannot be further analysed here, although in several investigations this species has been reported to be the primary invader (HARDING, 1989a; SOLHEIM, 1992a, 1992b). Until now the frequencies of ophiostomatoid fungi isolated from different points have not been analysed in a statistically reliable way. Odds ratios were found to be suitable for this purpose because there is no need for normal distribution assumptions, which are difficult to obtain from fungal species that demand different growth conditions.

According to the homogeneity test, ophiostomatoid species occurred equally in bark and sapwood, thus allowing the frequencies from two different sampling depths to be combined. The estimate of the odds ratio was nearly one, which also supported the previous observation.

4.3 Associations among the fungi

Based on the comparison of odds ratios, certain pairs of species were more likely than others to occur together; but considering the scarcity of some species in the data, speculation can be made only for the most frequent species. With *O. europhioides* and *O. penicillatum*, the presence of one species seemed to explain the occurrence of another, which supports the presence of a fungal complex. On the other hand, it is known that these species are related (SOLHEIM, 1986). Other pairs of ophiostomatoid species appeared to occur occasionally.

5 Conclusions

A major question was whether frequency distributions of ophiostomatoid species differed significantly depending on the method used to collect the beetles. The frequencies of fungi differed significantly only when fungi isolated from beetles collected in traps without vermiculite and those collected individually were compared. Thus, it can be seen from these data that with even slightly different sample sizes the frequencies of fungi arising from beetles might vary more. Previously there has been no support for this kind of speculation about fungal frequencies; for example, WRIGHT (1935) found no differences in frequencies of *Trichosporium symbioticum* n.sp. isolated from *Scolytus ventralis* LeConte collected individually and together.

Despite the fact that there is no unambiguous evidence of cross-contamination from other beetles due either to the presence or to the abundance of fungal species, this investigation shows that before further conclusions can be drawn from data collected with corresponding methods, the independence of the fungal frequencies should be tested. When beetles have been used for studies of fungal flora, the probability of cross-contamination by spores has not previously been analysed in detail. With the protocol presented here, possible sharing of fungal spores can be analysed reliably both one by one and statistically.

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References

BAKKE, A.; SÆTHER, T.; KVAMME, T., 1983: Mass trapping of the spruce bark beetle *Ips typographus*. Pheromone and trap technology. Medd. Nor. inst. skogforsk. **38,** 1–35.

CHRISTIANSEN, E., 1985: *Ips/Ceratocystis*-infection of Norway spruce: What is deadly dosage? Z. ang. Ent. **99,** 6–11.

DOWDING, P., 1969: The dispersal and survival of spores of fungi causing bluestain in pine. Trans. Br. mycol. Soc. **52,** 125–137.

FRANCKE-GROSMANN, H., 1963: Some new aspects in forest entomology. Ann. rev. Ent. **8,** 415–438.

FURNISS, M. M.; SOLHEIM, H.; CHRISTIANSEN, E., 1990: Transmission of Blue-Stain Fungi by *Ips typographus* (Coleoptera: Scolytidae) in Norway Spruce. Ann. Entomol. Soc. Am. **83,** 712–716.

HARDING, S., 1989a: Blue stain fungi associated with *Ips typographus* L. (Coleoptera: Scolytidae) in host trees of different vitality and at different beetle population levels. In: The influence of mutualistic blue stain fungi on bark beetle population dynamics. PhD thesis. Department of Zoology, Royal Veterinary and Agricultural University, Copenhagen **II**; 1–30.

—, 1989b: The pathogenicity of the blue stain *fungi Ophiostoma polonicum*, *Ophiostoma europhioides*, and *Ophiostoma penicillatum*, associated with *Ips typographus* L. (Coleoptera: Scolytidae), to Norway spruce. In: The influence of mutualistic blue stain fungi on bark beetle population dynamics. PhD thesis. Department of Zoology, Royal Veterinary and Agricultural University, Copenhagen **III**; 1–34.

—, 1989c: Rearing of spruce bark beetles, *Ips typographus* L. (Coleoptera: Scolytidae), without symbiotic blue stain fungi. In: The influence of mutualistic blue stain fungi on bark beetle population dynamics. PhD thesis. Department of Zoology, Royal Veterinary and Agricultural University, Copenhagen **IV**; 1–34.

HARRINGTON, T. C., 1993: Diseases of conifers caused by species of *Ophiostoma* and *Leptographium*. In: *Ceratocystis* and *Ophiostoma*, Taxonomy, Ecology and Pathogenicity. Ed. by WINGFIELD, M. J., SEIFERT, K. A., WEBBER, J. F. St. Paul, Minnesota: APS Press, 161–172.

HORNTVEDT, R.; CHRISTIANSEN, E.; SOLHEIM, H.; WANG, S., 1983: Artificial inoculation with *Ips typographus*-associated blue-stain fungi can kill healthy Norway spruce trees. Medd. Nor. inst. skogforskning **38,** 1–20.

KROKENE, P.; SOLHEIM, H., 1996: Fungal associates of five bark beetle species colonizing Norway spruce. Can. J. For. Res. **26,** 2115–2122.

MALLOCH, D.; BLACKWELL, M., 1993: Dispersal biology of the Ophiostomatoid Fungi. In: *Ceratocystis* and *Ophiostoma*, Taxonomy, Ecology and Pathogenicity. Ed. by WINGFIELD, M. J., SEIFERT, K. A., WEBBER, J. F. St. Paul, Minnesota: APS Press, 195–206.

MATHIESEN-KÄÄRIK, A., 1960: Studies on the ecology, taxonomy and physiology of Swedish insect-associated blue stain fungi, especially the genus *Ceratocystis*. Oikos. **11,** 1–25.

MEHTA, C. R.; PATEL, N. R., 1989, 1991: StatXact™. Statistical Software for Exact Nonparametric Inference. User Manual. Version 2. CYTEL Software Corporation. Cambridge.

MOSER, J. C.; PERRY, T. J.; SOLHEIM, H., 1989: Ascospores hyperphoretic on mites associated with *Ips typographus*. Mycol. Res. **93,** 513–517.

SOLHEIM, H., 1986: Species of Ophiostomataceae isolated from *Picea abies* infested by the bark beetle *Ips typographus*. Nord. J. Bot. **6,** 199–207.

—, 1992a: The early stages of fungal invasion in Norway spruce infested by the bark beetle *Ips typographus*. Can. J. Bot. **70,** 1–5.

—, 1992b: Fungal succession in sapwood of Norway spruce infested by the bark beetle *Ips typographus*. Eur. J. For. Path. **22,** 136–148.

—, 1993: Fungi associated with the spruce bark beetle *Ips typographus* in an endemic area in Norway. Scand. J. For. Res. **8,** 118–122.

UPADHYAY, H. P., 1981: A Monograph of *Ceratocystis* and *Ceratocystiopsis*. Athens: The University of Georgia Press. 175 pp.

—, 1993: Classification of the Ophiostomatoid Fungi. In: *Ceratocystis* and *Ophiostoma*, Taxonomy, Ecology and Pathogenicity. Ed. by WINGFIELD, M. J., SEIFERT, K. A., WEBBER, J. F. St. Paul, Minnesota: APS Press, 7–13.

WHITNEY, H. S., 1982: Relationships between Bark Beetles and Symbiotic Organisms. In: Bark beetles in North American Conifers. A System for the Study of Evolutionary Biology. Ed. by MITTON, J. B., STURGEON, K. B. Austin: University of Texas Press, 183–211.

WHITNEY, H. S.; BLAUEL, R. A., 1972: Ascospore dispersion in *Ceratocystis* spp. and *Europhium clavigerum* in conifer resin. Mycologia **64,** 410–414.

WRIGHT, E., 1935: *Trichosporium symbioticum*, n.sp., a wood-staining fungus associated with *Scolytus ventralis*. J. Agric. Res. **50,** 525–538.

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II

Ophiostomatoid fungi associated with the spruce bark beetle, *Ips typographus*, in post-epidemic areas in France

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Abstract
The species composition of ophiostomatoid fungi associated with *Ips typographus* was studied in the Vosges, Alps and Massif Central regions of France. In each region, damage caused by bark beetles has increased during recent years. For this study, beetles were collected individually by hand from freshly attacked trees and crushed in healthy *Picea abies* logs. Fungi were isolated from log phloem and sapwood, and identified. The most frequently found species were *Ophiostoma bicolor*, *O. penicillatum*, *Ceratocystiopsis minuta* and *Ceratocystis polonica*. Results are discussed in terms of differences between locations and in relation to previous investigations in which populations of spruce bark beetle have been sparse. The potential role of associated fungi in the population dynamics of the spruce bark beetle is discussed.

Key words: **associated fungi / *Ceratocystis* / *Ips typographus* / *Ophiostoma***

1 INTRODUCTION

European spruce (*Picea* spp.) forests suffer regularly from extensive outbreaks of the spruce bark beetle *Ips typographus* L. (Coleoptera: Scolytidae). Eurasian spruce bark beetles together with associated pathogenic fungi have killed millions of cubic metres of spruce in western and central Europe during recent years. In north-eastern France alone, damage has been as high as 100,000 m^3 in 1991, 212,500 m^3 in 1992 and 113,000 m^3 in 1993 (Boutte 1993, Nageleisen 1994, 1995). Severe beetle damage often follows heavy storm damage and windfall, *e.g.* as a result of the severe windstorm in December 1999.

Adults of the spruce bark beetle transport spores of blue-staining fungi both in the pronota and elytra and in the digestive tract (Furniss *et al.* 1990). When building breeding chambers and galleries, spruce bark beetles introduce the spores of *Ophiostoma* and *Ceratocystis* species into the phloem and cambium of Norway spruce, *Picea abies* L. Karsten. Together with associated fungi, spruce bark beetles can overcome the resistance of vigorous spruce trees. In the most harmful species, *Ceratocystis polonica* (Siemaszko) Moreau, pathogenicity is based on its ability to grow rapidly in moist wood through the tracheids and to disrupt water transport in the tree, finally leading to high mortality (Horntvedt *et al.* 1983, Christiansen 1985, Solheim 1988, Krokene and Solheim 1998, Kirisits 1998).

The main aim of this investigation was to describe the ophiostomatoid fungi associated with *I. typographus* in a locally high population level of spruce bark beetles. As further aim was to compare the fungal flora associated with spruce bark beetles collected from different regions. This information will provide us with useful elements for understanding the role of associated fungi as possible regulators of bark beetle epidemics.

2 MATERIAL AND METHODS

2.1 Study areas

Beetles were collected at the beginning of the main swarming period of the first generation, in late May and early June 1996, from three regions in France: Vosges, Alps and Massif Central (Figure 1, Table I). Two locations in each region were selected on the basis of previous large populations of beetles, and 50 beetles were collected at each location. At all locations, extensive spruce bark beetle damage has occurred in 1990–1995 (Boutte 1993, 1994). In Vosges, where two generations occur each year, the volume of dead Norway spruce varied between 1,200 and 5,900 m^3 in 1991–1995. In 1995, beetles were collected in pheromone traps and the total catch for three pheromone traps was 2,219 spruce bark beetles, thus indicating a declining trend (Office National des Forêts, Raon l'Etape). In Massif Central, at the Mézenc collecting site, the

high altitude reduces reproduction and only one generation of spruce bark beetles occurs annually. In Meygal, depending on weather conditions, 1–2 generations occur per year.

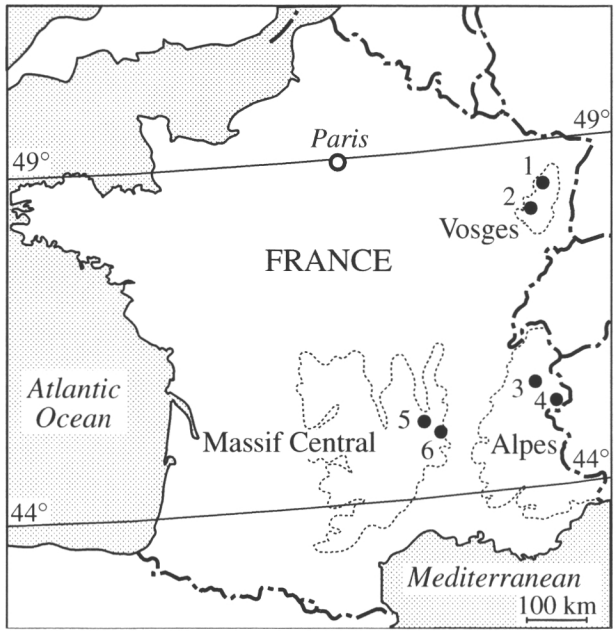

Fig. 1. Location of the *I. typographus* collecting areas. 1 = Val de Senones, 2 = Vologne, 3 = St Pierre de Belleville, 4 = St Michel de Maurienne, 5 = Meygal, 6 = Mézenc.

Table 1. Study areas used for collection of *I. typographus*.

Location	**Forest**	**Elevation** (m.s.l.)	**Stand age** (yrs)
1. Col de Praye, Vosges	Val de Senones	910	130
2. Tête de Nayemont, Vosges	Vologne	840	130
3. St Pierre de Belleville, Savoie	St Pierre de Belleville	1350	150
4. St Michel de Maurienne, Savoie	piles	450	150
5. Boussoulet, Haute Loire	Meygal	1300	120
6. Mont d'Alambre, Haute Loire	Mézenc	1480	120

2.2 Collecting beetles

At all locations, except St Michel de Maurienne, beetles were collected individually by digging out adult females and males with a knife and forceps from windblown Norway spruce trunks lying in the forest. In St Michel de Maurienne, beetles were collected in the Norway spruce trunks lying in a timber yard. Beetles were placed individually into sterile Eppendorf-test tubes. The equipment used for collection was sterilized after extraction of each individual. The logs had fallen during the previous winter and the beetles had just started to build galleries in them. Mostly the construction of nuptial chambers was completed and the mother galleries had been initiated. However, the mother galleries were less than 4 cm long. The collected beetles were stored individually at +4 °C in Eppendorf-test tubes for a maximum of three days before they were introduced into logs.

2.3 Inoculation, isolation and identification of fungi

Fungi were pre-cultivated in fresh uninfected Norway spruce bolts (one metre long, diameter 15 cm) according to the method described previously by Furniss *et al.* (1990). The bolts were brushed and the surfaces wiped with 70 % alcohol. To prevent drying, the ends of the bolts were dipped into melted paraffin. Then 25 beetles were introduced individually into each log through holes (5 mm diameter) bored previously with a cork-borer at the level of the cambium. After the beetle was introduced, the bark plugs were replaced and the beetles were crushed gently. In each log two control holes without beetles were made and treated similarly.

After 21 days of incubation at room temperature (+20 °C) reaction zones formed with phloem around each inoculation point. These reaction zones were then cut from the logs, wrapped in foil and stored at +4 °C for two weeks until used for isolations. Two phloem samples (50–60 mm^3) were taken from inside each necrotic zone, one at the border of the visible reaction and one at a distance of 15 mm. Two samples were also taken from a depth of 1 mm in the sapwood. When the reaction zones were less than 20 mm long, all four samples were taken from the edge of the visible reaction zone. When reaction zones were more than 150 mm long, six samples were taken, four from the phloem and two from the sapwood. A total of 1,221 primary samples were taken around the inoculation points. Samples were cultivated in petri dishes (2 % malt and 1.4 % agar medium) at room temperature. Occasionally, pieces of fresh autoclaved phloem or sapwood of Norway spruce were added to the dishes to promote formation of sexual stages. A type specimen of each ophiostomatoid fungi was purified by transferring mycelium or spores from individual colonies to fresh malt agar medium.

Reproduction structures of the fungi were prepared with lacto-fuchsin, lactic acid or cotton blue for identification. Fungal structures were compared with the species descriptions given in the literature (Siemaszko 1939, Davidson 1953, Mathiesen-Käärik 1953, Wright and Cain 1961, Kendrick 1962, Davidson *et al.* 1967, Upadhyay 1981, De Hoog and Scheffer 1984, Solheim 1986, Kirisits 1996, Yamaoka *et al.* 1997).

2.4. Statistics

Frequencies of ophiostomatoid species were analysed with the Kruskal-Wallis test. Due to the sparseness of the observed frequencies, the data were analysed with StatXact™ Version 2.11 software, a statistical package for exact nonparametric inference (Mehta and Patel 1989, 1991). Since the data sets were too large for exact calculation of p-value, the Monte-Carlo estimates of the p-value were computed by generating 100,000 tables. The level of significance in the tests was $p < 0.01$.

3 RESULTS

The most common and consistently occurring species were *Ophiostoma bicolor* Davidson & Wells, *O. penicillatum* (Grosm. Siemaszko), *Ceratocystiopsis minuta* (Siemazko) Upadhyay & Kendrick and *C. polonica*. Other frequently isolated species were *O. piceaperdum* (Rumbold) Arx and *O. ainoae* Solheim (Table II). *O. piceae* (Münch) H. & P. Sydow and *O. cucullatum* Solheim were isolated occasionally. There was no visible staining on any of the control inoculations, and no ophiostomatoid fungi were detected in the control inoculations.

Furthermore, an unidentified strain of ophiostomatoid species was isolated which differed from known species by having perithecia formed in mixed cultures within the malt extract substratum, perithecia black, 160–230 µm in diameter, necks 3,840–4,800 µm long, tapered, dark brown to black, 40–50 µm wide at the base, 20 µm wide at the apex, and ostiolar hyphae absent. Ascospores were cucullate with a hyaline wall, released in a slimy mass. No conidia or conidiophores were seen.

When the frequencies of nine ophiostomatoid species were compared simultaneously at six beetle-collection locations, the Kruskal-Wallis analysis of variance indicated a highly significant difference between locations ($\chi^2 = 29.04$, $df = 8$, asymptotic p-value = 0.0003). When the five most frequent species (*C. minuta*, *C. polonica*, *O. bicolor*, *O. piceaperdum* and *O. penicillatum*) were compared, there was also a significant difference between locations (locations ($\chi^2 = 16.86$, $df = 4$, asymptotic p-value = 0.0021).

Table 2. Frequencies of occurrence of ophiostomatoid fungi associated with *I. typographus* collected at six locations in France. Locations presented in Table 1. n = 50 beetles per location.

	Vosges		Alps		Massif Central	
	Senonne	**Vologne**	**Belleville**	**Maurienne**	**Meygal**	**Mézenc**
C. minuta	62	36	36	30	28	24
C. polonica	40	32	22	50	42	30
O. ainoae	2	10	28	12	24	10
O. bicolor	54	74	26	44	66	42
O. cucullatum	0	0	0	0	2	0
O. piceaperdum	20	34	30	10	16	28
O. penicillatum	40	40	24	26	60	40
O. piceae	8	12	10	12	8	2
"Long-necked"	2	0	6	4	8	0
Pesotum spp.	36	28	46	46	50	58
Leptographium spp.	2	2	0	0	0	0
Primary isolations	204	204	202	202	205	204

4 DISCUSSION

The blue-stain fungi *Ceratocystis polonica*, *Ophiostoma bicolor*, *O. europhioides* Wright & Cain (Solheim) and *O. penicillatum* have been reported to be associated with *I. typographus*, occurring with varying frequency in different environmental conditions and investigations (Solheim 1986, 1992, 1993, Harding 1989, Krokene and Solheim 1996, Viiri 1997). O. *europhioides* was recently synonomised with *O. piceaperdum*, since they cannot be distinguished on the basis of morphology (Jacobs *et al.* 2000). Some characteristics of the long-necked ophiostomatoid species isolated here are the same as the species characteristics of *O. piceaperdum*, but possible synonymy needs to be clarified in more detailed studies of teleomorph and anamorph morphology.

The most common and consistently occurring fungus in this study was *O. bicolor*, which was recovered in Vologne from 74 % of the bark beetles examined. At nearly all locations, *C. minuta*, *O. ainoae*, *O. bicolor*, *O. penicillatum* and *O. piceaperdum* occurred at higher frequencies than recorded

from the low population density areas of *I. typographus* (Solheim 1993, Viiri 1997). The following ophiostomatoid species have been reported to be associated with *Ips sexdentatus* Boern in France: *C. minuta*, *O. bicolor*, *O. brunneo-ciliatum* Mathiesen-Käärik, *O. europhioides*, *O. ips* (Rumbold) Nannf., *O. piceae* and *O. minus* (Hedgcock) H. & P. Sydow (Levieux *et al.* 1989, Lieutier *et al.* 1989, 1991). Correspondingly, the species found to be associated with *Ips acuminatus* Gyll are *O. brunneo-ciliatum*, *O. ips*, *O. minus* and *Ceratocystiopsis minima* (Olchow. and Reid) (Lieutier *et al.* 1991).

According to surveys made in previous years, in all sampling areas, especially in Vosges and Massif Central, the population levels of the spruce bark beetle were high. This resulted in numerous spontaneous attacks on spruce trees in these areas. Pheromone trapping, although done only in Vosges, showed declining population size already during the year of beetle sampling. Thus the isolated fungal flora constantly corresponded to a beetle population in the post-epidemic phase. According to Weslien *et al.* (1989), fewer than 15,000 spruce bark beetles in a group of three traps corresponds to a low population level. Hübertz *et al.* (1991) caught 3,400–12,000 individuals and Valkama *et al.* (1997) at highest 14,000 individuals per season with a group of three traps during a period when the beetle population was low.

According to Yamaoka *et al.* (1997), the technique used to isolate ophiostomatoid fungi from various niches can greatly affect the frequencies of occurrence. Thus when results are compared to those of other authors, discrepancies in fungal frequencies may be partly due to differences in methods of sampling and isolation. In this study, however, the fungal flora differed significantly between locations.

Both *C. polonica* and *O. piceaperdum* have been suggested to play a special role in the population dynamics of the spruce bark beetle (Solheim 1993, Harding 1989). It has been proposed that during endemic periods when beetles utilise dead trees and timber for breeding, pathogenic species can be replaced by less harmful ones. In Norway, the frequency of *C. polonica* has been low during periods of low population level when beetles use dead trees and timber, whereas the frequency has been higher during the epidemic phase when living trees are attacked (Solheim 1992, 1993, Krokene and Solheim 1996). The previous finding that the frequency of the pathogenic species, *C. polonica* (Viiri 1997), in the endemic population of spruce bark beetle is low does not conflict with the fact that associated pathogenic fungi can regulate the damage by spruce bark beetles. Our results are thus in agreement with those suggesting that pathogenic species can be replaced by other species during endemic periods. Furthermore, they support the idea that the role of the associated fungi may vary under different environmental conditions.

The success or failure of bark beetle attacks on living trees is ultimately determined by the beetle-fungus-host tree interaction. Owing to conflicting results concerning frequency and pathogenicity, genetic variation within the

species *O. piceaperdum* and *C. polonica* needs to be clarified (Harding 1989, Kirisits and Angelberger 1999, Krokene and Solheim 2001). The pathogenicity of geographically different strains of *O. piceaperdum* and *C. polonica* should be tested.

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REFERENCES

Boutte B., Le typographe de l'épicéa, in: Département de la Santé de Forêts, La Santé des Forêts [France] en 1992, Ministère de l'agriculture, de la pêche et de l´alimentation, (1993) 12-13.

Boutte B., Le typographe de l'épicéa, in: Département de la Santé de Forêts, La Santé des Forêts [France] en 1993, Ministère de l'agriculture, de la pêche et de l´alimentation, (1994) 14-17.

Christiansen, E., *Ips/Ceratocystis*-infection of Norway spruce: what is deadly dosage? Z. ang. Ent. 99 (1985) 6-11.

Davidson R.W., Two common lumber-staining fungi in the western United States, Mycologia 45 (1953) 579-586.

Davidson R.W, Francke-Grosmann H., Käärik A., A Restudy of *Ceratocystis penicillata* and report of two American species of this genus from Europe, Mycologia 59 (1967) 928-932.

Furniss M.M., Solheim H., Christiansen E., Transmission of blue-stain fungi by *Ips typographus* (Coleoptera: Scolytidae) in Norway spruce, Ann. Entomol. Soc. Am. 83 (1990) 712-716.

Harding S., The influence of mutualistic blue stain fungi on bark beetle population dynamics, Ph.D. thesis, Department of Zoology, Royal Veterinary and Agricultural University, Copenhagen, 1989.

De Hoog G.S., Scheffer R.J., *Ceratocystis* versus *Ophiostoma*: A Reappraisal, Mycologia 76 (1984) 292-299.

Horntvedt R., Christiansen E., Solheim H., Wang S., Artificial inoculation with *Ips typographus*-associated blue-stain fungi can kill healthy Norway spruce trees, Rep. of Norwegian For. Res. Inst. 38 (1983) 1-20.

Hübertz, H., Larsen, J.R., Bejer, B., Monitoring spruce bark beetle (*Ips typographus*) populations under non-epidemic conditions, Scand. J. For.

Res. 6 (1991) 217-226.

Jacobs, K., Wingfield, M.J., Crous, P.W., *Ophiostoma europhioides* and *Ceratocystis pseudoeurophioides*, synonyms of *O. piceaperdum*, Mycol.Res. 104 (2000) 238-243.

Kendrick W.B., The *Leptographium* complex. Verticicladiella Hughes, Can. J. Bot. 40 (1962) 629-770.

Kirisits, T., Untersuchungen über die Vergesellschaftung von Bläuepilzen (*Ophiostoma/Ceratocystis* spp.) mit den rindenbrütenden Fichtenborkenkäfern *Ips typographus* L., *Pityogenes chalcographus* L. und *Hylurgops glabratus* Zett. in Österreich. Master thesis, Insitut für Forstentomologie, Forstpathologie und Forstschutz, Universität für Bodenkultur, Wien. 175 p. 1996.

Kirisits, T., Pathogenicity of three blue-stain fungi associated with the bark beetle *Ips typographus* to Norway spruce in Austria, Österr. Z. Pilzk. 7 (1998) 191-201.

Kirisits, T. & Anglberger, H., Report on a strain of the pathogenic blue-stain fungus *Ceratocystis polonica* with low virulence, Österr. Z. Pilzk. 8 (1999) 157-166.

Krokene P., Solheim H., Fungal associates of five bark beetle species colonizing Norway spruce, Can. J. For. Res. 26 (1996) 2115-2122.

Krokene P., Solheim H., Pathogenicity of four blue-stain fungi associated with aggressive and nonaggressive bark beetles, Phytopathology 88 (1998) 39-44.

Krokene P., Solheim H., Loss of pathogenicity in the blue-stain fungus *Ceratocystis polonica*, Plant Pathology 50 (2001) 497-502.

Levieux, J., Lieutier, F., Moser, J.C., Perry, T.J., Transportation of phytopathogenic fungi by the bark beetle *Ips sexdentatus* Boerner and associated mites, J. App. Ent. 108 (1989) 1-11.

Lieutier, F., Garcia, J., Yart, A., Vouland, G., Pettinetti, M. & Morelet, M., Ophiostomatales (Ascomycètes) associées à *Ips acuminatus* Gyll (Coleoptera: Scolytidae) sur le pin sylvestre (*Pinus sylvestris* L) dans le Sud-Est de la France et comparaison avec *Ips sexdentatus* Boern, Agronomie 11 (1991) 807-817.

Lieutier, F., Yart, A., Garcia, J., Ham, M.C., Morelet, M. & Levieux, J., Champignons phytopathogènes associés à deux coléoptères scolytidae du pin sylvestre (*Pinus sylvestris* L.) et étude préliminaire de leur agressivité envers l'hôte, Ann. Sci. For. 46 (1989) 201-216.

Mathiesen-Käärik A., Eine Übersicht über die gewöhnlichsten mit Borkenkäfern assoziierten Bläuepilze in Schweden und einige für Schweden neue Bläuepilze, Medd. Stat. skogsforskningsinst. 43 (1953) 1-74.

Mehta, C.R., Patel, N.R., StatXact™. Statistical Software for Exact Nonparametric Inference. User Manual. Version 2. CYTEL Software Corporation. Cambridge, MA. 1989, 1991.

Nageleisen L-M., Lutte Contre les Scolytes en 1993, in: Information technique

N°21, Dèpartement de la Santé des Forêts Nord-Est, Ministère de l'agriculture, de la pêche et de l´alimentation, 1994.
Nageleisen L.-M., Epicéa: le typographe, in: Bilan Département de la Santé des Forêts Nord-Est 1994, Ministère de l'agriculture, de la pêche et de l´alimentation, 1995.
Siemaszko W., Zespoły grzybów towarzyszących kornikom Polskim, Planta Polonica 7 (1939) 1-54.
Solheim H., Species of Ophiostomataceae isolated from *Picea abies* infested by the bark beetle *Ips typographus*, Nord. J. Bot. 6 (1986) 199-207.
Solheim H., Pathogenicity of some *Ips typographus*-associated blue stain fungi to Norway spruce, Rep. of the Norwegian For. Res. Inst. 40 (1988) 1-11.
Solheim H., The early stages of fungal invasion in Norway spruce infested by the bark beetle *Ips typographus*, Can. J. Bot. 70 (1992) 1-5.
Solheim H., Fungi associated with the spruce bark beetle *Ips typographus* in an endemic area in Norway, Scand. J. For. Res. 8 (1993) 118-122.
Upadhyay H.P., A Monograph of *Ceratocystis* and *Ceratocystiopsis*, The University of Georgia Press, Athens, Georgia, 1981.
Valkama, H., Räty, M., Niemelä, P., Catches of *Ips duplicatus* and other non-target Coleoptera by *Ips typographus* pheromone trapping, Entomol. Fenn. 8 (1997) 153-159.
Viiri H., Fungal associates of the spruce bark beetle *Ips typographus* L. (Col. Scolytidae) in relation to different trapping methods, J. Appl. Ent.121 (1997) 529-533.
Weslien, J., Annila, E., Bakke, A., Bejer, B., Eidmann, H.H., Narvestad, K., Nikula, A. & Ravn, H.P., Estimating risks for spruce bark beetle (*Ips typographus*) damage using pheromone baited traps and trees. Scand. J. For. Res. 4 (1989) 87-98.
Wright E.F., Cain R.F., New species of the genus *Ceratocystis*, Can. J. Bot. 39 (1961) 1215-1230.
Yamaoka Y., Wingfield M.J., Takahashi I., Solheim H., Ophiostomatoid fungi associated with the spruce bark beetle *Ips typographus* f. *japonicus* in Japan, Mycol. Res. 101 (1997) 1215-1227.

III

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ORIGINAL ARTICLE

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Induced responses in stilbenes and terpenes in fertilized Norway spruce after inoculation with blue-stain fungus, *Ceratocystis polonica*

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Abstract In conifers, attacks by bark beetles and associated pathogenic fungi cause an induced wound response, which is characterized by accumulation of antifungal compounds and morphological changes that aid wound healing. In this article the stilbene and terpene concentrations of Norway spruce phloem were monitored as symptoms of induced wound responses in relation to changed nutrient conditions caused by fertilization. Plots of mature Norway spruce were fertilized with N, P or NPK. One year after fertilization the trees were artificially infected with *Ceratocystis polonica*, a pathogenic fungus associated with the bark beetle *Ips typographus*. The response of stilbenes to fungal inoculation was mainly qualitative. The concentration of stilbene glycosides in the phloem decreased, and in the immediate vicinity of the site of fungal inoculation, stilbene glycosides were less frequent than in mechanically wounded or unwounded phloem. Corresponding stilbene aglycones were most frequent inside the reaction lesion. The concentration of total stilbene aglycones near the inoculation site was significantly lower in N-fertilized trees than in unfertilized trees. Fungal inoculation caused a strong quantitative response in terpenes. The total terpene concentration of the phloem increased significantly, to almost 100 times greater near the inoculation site compared to the constitutive values. N fertilization significantly reduced the total terpene and total stilbene aglycone concentrations near the inoculation sites. Thus, N fertilization may reduce the ability of Norway spruce to defend itself against fungal pathogens.

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Introduction

Many bark beetles distribute spores of fungi laterally or internally in the digestive tract while constructing breeding chambers and galleries in the phloem of their host trees. As they grow, fungal hyphae suppress water transportation in the host, causing discolouration of wood and helping the beetles to kill the trees. New generations of beetles transport fungi to new host trees. For many species of fungi, transmission by insects is vital, and special associations have arisen between insects and fungi. Most fungi associated with the Scolytidae belong to the genus *Ceratocystis* sensu stricto De Hoog and Scheffer, Ophiostoma H. and P. Sydow and their anamorph genera (Wingfield et al. 1993; Krokene and Solheim 1996). These fungi are adapted to dispersal by insects: elongated ascocarps bear ascospores at the apices of their necks, which may be protected by a gelatinous matrix (Wingfield et al. 1993).

From the human point of view, a major part of the symbiosis between bark beetles and associated fungi is their joint action to overcome the resistance mechanisms of their host trees. Some fungi are pathogenic and, when mass inoculated into trees, are able to kill healthy trees (Christiansen 1985). When phloem is infected by bark beetles and associated pathogenic fungi, a resistance reaction may be initiated that is characterized by rapid desiccation of cells and necrosis around the site of the wound (Berryman 1972). The lesion surrounding the attacked site is impregnated with secondary metabolites, i.e. phenolic and terpenoid compounds, and isolated by the formation of wound periderm, callus tissue and traumatic resin cavities at the cambium-sapwood interface (Reid et al. 1967; Berryman 1969; Woodward and Pearce 1988a, 1988b; Delorme and Lieutier 1990). The co-determinants of conifer resistance to bark beetle-fungus attack have been tentatively identified as the primary, or

resident, resin canal system and the hypersensitive reaction, which functions as wound cleansing, containment of infection and wound healing (Berryman 1972). Induced wound responses can start as a result of mechanical wounding, but formation of traumatic secondary compounds and extension of the lesion require fungal infection or boring activity by beetles (Lieutier et al. 1989; Franceschi et al. 1998).

Accumulation of monoterpenes in high concentrations is characteristic of the defensive response in several conifers; α-pinene, β-pinene, myrcene, terpinolene, sabinene, Δ-3-carene or limonene, in particular, have been shown to increase in response to inoculation with fungi (Russell and Berryman 1976; Raffa and Berryman 1982a; Gershenzon 1994). This resinous material is stored in specialized resin ducts or granular cells (Berryman 1969; Franceschi et al. 2000; Nagy et al. 2000). Monoterpenes prevent growth of pathogenic fungi (Cobb et al. 1968; Delorme and Lieutier 1990) and repel or kill bark beetles (Raffa et al. 1985; Bridges 1987; Raffa and Smalley 1995). Thus, a change in any of these compounds may be important for the defence of the tree against beetles and fungi.

The bark of many conifers also contains antifungal phenolic compounds, i.e. stilbenes and flavonoids. In healthy phloem of Norway spruce, *Picea abies* (L.) Karst., stilbenes typically occur as glycosides (Brignolas et al. 1995). The main constitutive stilbene glycosides in the bark of *Picea* species are astringin and isorhapontin (Woodward and Pearce 1988a; Solhaug 1990; Toscano Underwood and Pearce 1991a, 1991b; Lindberg et al. 1992). In vitro these compounds have antifungal properties (Woodward and Pearce 1988a). Rapid accumulation of stilbenes in response to injury or fungal infection is considered to be an active defence response (Nicholson and Hammerschmidt 1992; Schultz et al. 1992). Phenolics are formed in situ from carbohydrates in phloem parenchyma cells (Hart 1981; Franceschi et al. 1998) via the shikimate/phenylpropanoid-acetate pathways. The proportion and type of synthesized stilbenes (Brignolas et al. 1995, 1998) and other secondary compounds are controlled by the plant genotype. However, physiological conditions also affect the ability of trees to produce secondary compounds (Raffa and Berryman 1982b; Klepzig et al. 1995).

Brignolas et al. (1998) proposed that the induced response in spruce phloem after fungal inoculation involves two phases. The first of these is the tree's response to wounding, which is characterized mainly by both an increase in the (+)catechin concentration and a slow decrease in the protein-precipitation capacity of wound extract (or tannins). Thereafter, a violent fungus-dependent reaction occurs, which is characterized by a strong decrease in the stilbene glycoside concentration, delayed appearance of the corresponding aglycones and a strong decrease in both the (+)-catechin concentration and the capacity to precipitate protein. This agrees with the nonspecific healing model proposed by Woodward and Pearce (1988b). In their model, stilbene glycosides, coupled with terpenes, provide the first line of defence after the bark surface is breached. The action of β-glycosidases produced by the fungi close to the wound releases stilbene aglycones, thus inhibiting the growth of most pathogens. The stilbenes and terpenes provide the first barrier to invasion, allowing time for the formation of necrophylactic periderm and isolating the necrotic tissues.

Balanced availability of nutrients is assumed to affect the vigour of trees and their ability to resist attack by bark beetles and associated fungi. As hypersensitivity is an active metabolic process, the reaction capacity and speed must be affected by the physiological vigour of the tree (Berryman 1972). The number of bark beetle attacks on a tree may also modify the resistant response. Each attack severs resin ducts, causes the expenditure of energy in hypersensitive reaction, and reduces the area of functional phloem and sapwood (Berryman 1969, 1972). The growth/differentiation balance hypothesis predicts a physiological trade-off between plant growth and differentiation processes. When environmental conditions are favourable, resources are generally allotted by giving priority to vegetative growth over secondary metabolism and storage (Herms and Mattson 1992; Lerdau et al. 1994). Even moderate shortages of nutrients or water slow growth processes considerably. Net photosynthesis, however, is not as sensitive to limitations in resources (Chapin 1980). Thus, when a moderate nutrient deficiency imposes sink limitations upon growth, the growth is limited more by nutrient availability than by photosynthate production. Therefore, carbohydrates accumulate in "excess" of growth requirements and are allocated to the production of C-based secondary metabolites, e.g. phenolics and terpenoids (Gershenzon 1994). If growth is stimulated more strongly than photosynthesis, then concentrations of carbohydrates and C-based secondary compounds decline and the production of N-based compounds increases. These predictions are known as the carbon/nutrient balance hypothesis (CNB). More specifically, concentrations of C-based secondary metabolites are positively correlated with the C/N ratio of the plant (Bryant et al. 1983; Herms and Mattson 1992).

The aim of the present experiment was to test for changes in the C-based secondary compounds, such as stilbenes and terpenes, in Norway spruce after mechanical wounding and artificial inoculation with *Ceratocystis polonica* Siem. Moreau, a pathogenic fungus transmitted by the spruce bark beetle *Ips typographus* L. (Solheim 1988; Krokene and Solheim 1996). In addition, the extent and formation of induced wound responses were studied in relation to increased nutrient concentration and growth caused by fertilization treatments. Results concerning carbohydrates and growth of experimental trees are reported in a companion paper.

Materials and methods

Experimental area and trees

The study area was located in a *Myrtillus*-type spruce forest in Karttavuori, Vesijako, southern Finland. The stand was of natural origin and the selected trees were mature (age ca. 80 years) Nor-

way spruce. Four plots of 0.5 ha were marked, and fertilization treatments were randomly applied to the plots. There was a 10- to 15-m-wide buffer zone between plots. Fertilizers N (173 kg ha^{-1} $year^{-1}$; NH_4-N 13.5%; NO_3-N 13.5%; Ca 3%; Mg 1%; S 3%; B 0.02%), P (41 kg ha^{-1} $year^{-1}$; total P 9%; water-soluble P 8%; Ca 20%; S 11%) and NPK (800 kg ha^{-1} $year^{-1}$; NH_4-N 11%; NO_3-N 7%; total P 5%; water-soluble P 3.6%; K 10%; Ca 3%; Mg 0.5%; S 3%; B 0.02%; Se 0.001%) were spread by hand at the beginning of the growing season in May 1993. The control treatment was an unfertilized plot. Due to space limitations, we used a single plot for each fertilization treatment and one control. Thus, our experimental design was pseudoreplicated (Hurlbert 1984); this is taken into account in the interpretation of results, where fertilization treatments are compared with the control, not with each other. From each plot 30 trees that were free of visible wounds, a total of 120 trees, were selected. Trees were chosen so that there were 97 dominants, 22 co-dominants and one intermediate tree. Two trees that were infected with root-rot and two that had been attacked by *I. typographus* were omitted from the analysis. At the end of the experiment, the sample trees averaged 34±0.5 cm in diameter at breast height and 28±0.2 m in total height.

Fungal inoculations

In June 1994 a culture of blue-stain fungus, *C. polonica*, on 2% malt extract agar in Petri dishes was inoculated into ten trees per fertilization treatment. The fungal strain had been isolated from Norway spruce that had been infected naturally with *I. typographus* in Tuusula, southern Finland. The fungus was inoculated into the phloem with a cork-borer (diameter 5 mm), and the bark plug was re-inserted into the hole. Each tree received four evenly spaced inoculations made at the cardinal points of the compass 1.3 m above ground. For mechanical wounding, the trees were injured with a cork borer in the same way as for inoculation, but without the fungus and malt agar. Ten trees per fertilization treatment were wounded mechanically.

In the middle of August, after a 2-month incubation period, the dead rhytidome was removed carefully with a sharp knife around each inoculation point so that the reaction lesion was visible. The vertical length of the lesion and the bark, sapwood and phloem thicknesses, were measured. Lesion length includes only the area outside the bark plug. Around each fungal inoculation site, four phloem samples were taken with a cork-borer (diameter 15 mm) to the level of the cambium: two from the distal ends of the visible reaction lesion (henceforth called "far" samples) and two immediately above and below the site of inoculation (henceforth called "near" samples). Two samples were taken from around the site of mechanical wounding, one above the wounding site and the other below the wounding site. In cases where there was visible lesion formation around the mechanical wounding (necrotic area more than 3 mm at the upper and lower side of the inoculation site, n=11), these necrotic tissues were included as part of the samples. Two samples per tree of phloem from intact trees were used as unwounded controls. All samples were immediately placed on dry ice and stored at –20°C until analysed. The fungus was not re-isolated from inoculation sites, but all reaction lesions were visually distinct from each other.

Extraction and analysis of stilbenes

Phloem samples were ground in liquid N. Then 100 mg ground phloem powder was extracted with 2 ml of 80% v/v EtOH. As internal reference compounds, 50 µl (10,000 ppm) rhapontin and 50 µl (5,000 ppm) diethyl stilbestrol (Sigma) were added to the extracts. The extraction liquid was placed in an ultrasonic bath for 45 min, after which the liquid was extracted overnight. The extract was shaken on an orbital shaker and then centrifuged (4,332 g; 20 min^{-1}); 500 µl of supernatant was evaporated to dryness in a stream of N. Dried samples were silylated for 2 h at room temperature with 400 µl silylating reagent prepared from 100 ml dry pyridin and 21 ml trimethylsilyl-imidazole (Fluka) and preserved in complete dryness over silica gel. The silylated samples were shaken and preserved at –20°C until analysed.

Stilbenes were analysed with a gas chromatography (Hewlett-Packard 5890) mass spectrometry (Hewlett-Packard 5988A) system with a HP-5 (Hewlett-Packard) capillary column (25 m×0.2 mm×0.3 µm). Split injection was used. The carrier gas was helium; the column head pressure was 100 kPa and the split flow was 25 ml min^{-1}. The initial temperature of the oven was 110°C, increasing at 10°C min^{-1} to a final temperature of 300°C. Stilbene glycosides were quantified by the response of rhapontin, and stilbene aglycones were quantified by using the response factor of diethyl stilbestrol and resveratrol. Identifications of compounds were confirmed by mass spectra. Results are expressed as micrograms of stilbenes per milligram of fresh phloem.

Extraction and analysis of mono- and sesquiterpenes

One hundred milligrams of phloem powder obtained by grinding the phloem in liquid N was extracted with 2 ml hexane. The hexane contained 200 ppm each of the following three internal standards: $C_{10}H_{21}Cl$, $C_{14}H_{29}Cl$ and $C_{18}H_{37}Cl$ (Fluka). The samples were extracted in an ultrasonic bath for 30 min and left in test tubes overnight. The hexane extracts were shaken and centrifuged (4,332 g; 20 min^{-1}); and the extracted samples were stored at –20°C until analysed.

Mono- and sesquiterpenes were analysed with the previously described gas chromatography system and the mass spectrometry system using an NB-351 (HNU-Nordion, Helsinki, Finland) capillary column (25 m×0.2 mm×0.3 µm). The programme started at 60°C (splitless injection, 0.5 min), rising by 5°C min^{-1} to a final temperature of 230°C. The analysis time was 40 min, the carrier gas was helium, and the split flow rate was 20 ml min^{-1}. Monoterpenes were identified and quantified using the retention and mass spectral data of authentic model compounds. Sesquiterpenes were identified according to the method of Pohjola (1993) and quantified according to the response factor of caryophyllene. Model compounds were obtained from the Pharmacognosy Division, Department of Pharmacy, University of Helsinki, Finland. The results are expressed as micrograms of terpenes per milligram of fresh phloem.

Statistical analysis

Univariate ANOVA was used to examine overall differences between inoculation methods and between inoculation directions. The means of chemical compounds calculated from opposite sides of the inoculation site were used in the analysis. For mechanically wounded trees and unwounded controls, the means of two sampling sites were used. Detection frequency is the mean (%) of all

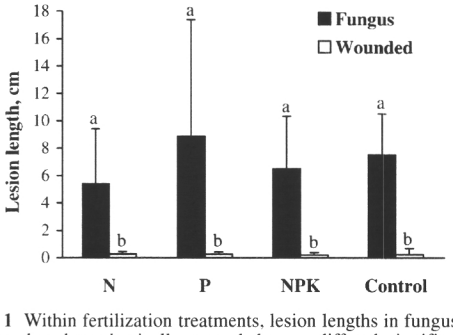

Fig. 1 Within fertilization treatments, lesion lengths in fungus-inoculated and mechanically wounded trees differed significantly from each other at the 5% level; these differences are indicated by *different letters*

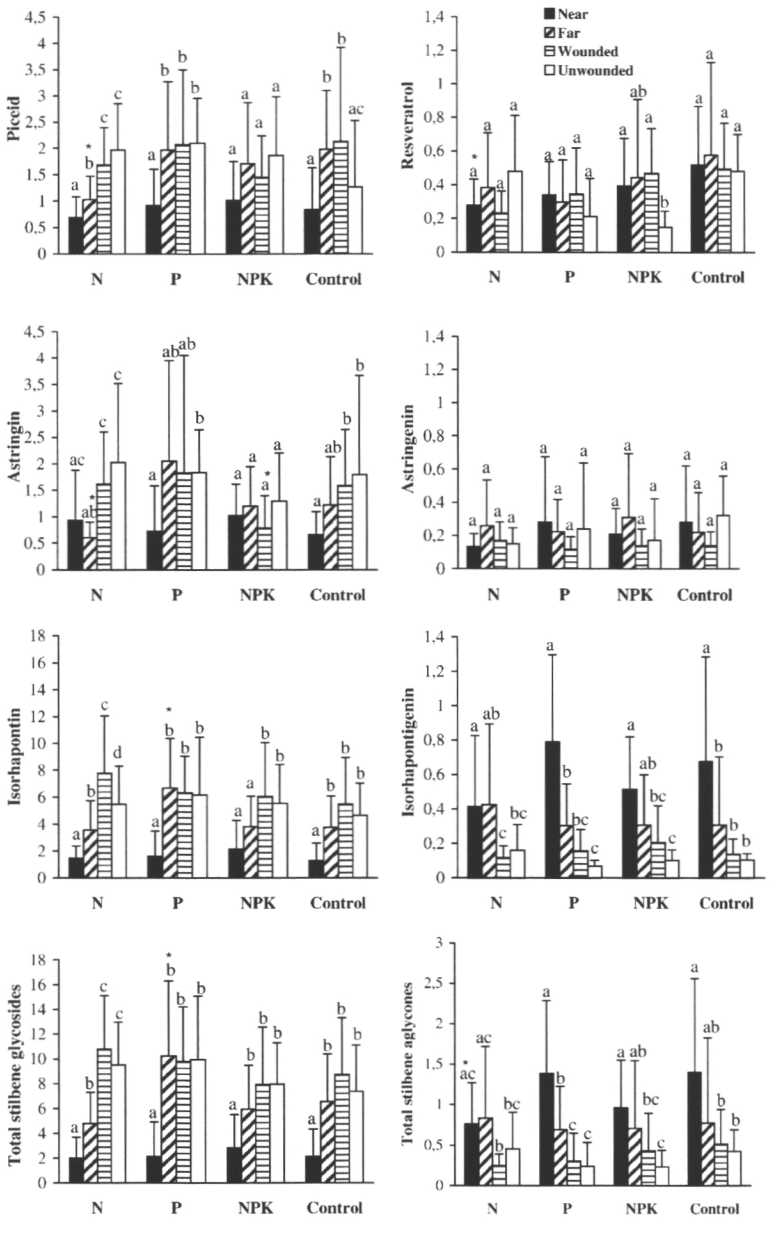

Fig. 2 Stilbene concentrations (μg mg^{-1} fresh phloem tissue, mean±SD) in *Picea abies* phloem inoculated with *Ceratocystis polonica*, mechanically wounded and unwounded phloem (see Materials and methods regarding sampling), grouped according to fertilization. Values indicated with an *asterisk* at the end of a bar differ significantly from corresponding unfertilized phloem at the 5% level. Within fertilization treatments, bars with *different letters* differ significantly from each other at the 5% level

samples in which the substance in question was detected. The Dunnett-test (Myers and Well 1995) was used to examine differences between fertilized plots and the unfertilized control and within fertilization treatments. Homogeneity of the variances was tested with Levene's test (Snedecor and Cochran 1980). If the assumption of homogeneity was not shown to be true (Jeffers 1960), nonparametric Dunnett-C tests were used. The values presented in the tables and figures are untransformed means, and if not mentioned otherwise, the significance level is 5%.

Results

Lesion length

Fungal inoculation produced a necrotic lesion around the inoculation site, the vertical length of which was 7.1±0.8 cm (mean of all fertilizations±SE; n=39). In me-

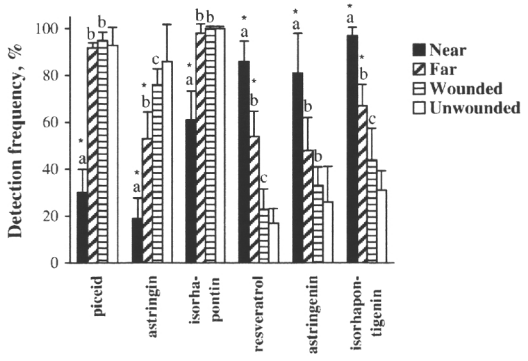

Fig. 3 Detection frequency of stilbene compounds (%) in *P. abies* phloem after inoculation with *C. polonica*, in mechanically wounded and unwounded phloem (see Materials and methods for sampling information). Each fertilization treatment and the control were combined. Values indicated with an *asterisk* at the end of a bar differ significantly from unwounded phloem at the 5% level. For the same compound, bars labelled with a *different letter* differ significantly from each other at the 5% level

chanically wounded trees the vertical length of the lesion around the inoculation sites was 0.2±0.3 cm (n=38), and horizontal growth was <0.3 cm. The difference in lesion length between fungal inoculation and mechanical wounding was highly significant (Fig. 1). The lesions appeared to be longest in P-fertilized trees and shortest in N-fertilized trees. After fungal inoculation the difference between fertilization treatments was nearly significant (F=2.47, df=3, P=0.064).

Stilbenes

Concentrations of the stilbene glycosides (piceid, astringin, isorhapontin) and their aglycones (resveratrol, astringenin and isohapontigenin) in relation to inoculation and fertilization are shown in Fig. 2. Near the inoculation site, concentrations of individual glycosides and total glycosides were lower than in unwounded phloem regardless of treatment (Fig. 2). The total stilbene glycoside concentration showed a negative and significant correlation with the total terpene concentration near the inoculation site (r=–0.493, n=33, P<0.01), and at the outer border of the lesion (r=–0.433, n=39, P<0.01). The

Fig. 4 Composition of the major monoterpenes and the sesquiterpene germacrene (%, mean±SD) grouped according to fertilization. Labels as in Fig. 2

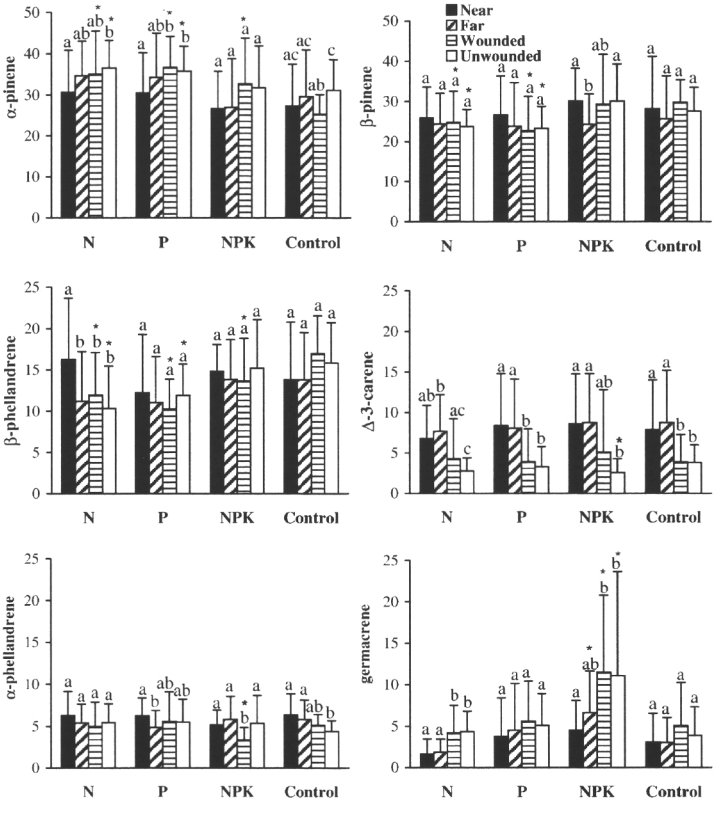

Table 1 Quantitative changes in the terpene fraction (μg mg^{-1} fresh phloem) of *Picea abies* phloem inoculated with *Ceratocystis polonica*, mechanically wounded and unwounded phloem. All fertilization treatments were pooled. Samples were taken around the fungal inoculation sites. *Near* Mean of two samples from the areas immediately above and below the site of inoculation, *Far* mean of two samples from the distal ends of the visible reaction lesion

	Near	Far	Wounded	Unwounded
Tricyclene	0.172***	0.022***	0.004	0.004
α-Pinene	26.469***	3.407***	0.326	0.351
Fenchene	0.012***	0.009**	0.003	0.003
Camphene	0.560***	0.080***	0.007	0.008
β-Pinene	27.878***	2.913***	0.278	0.278
Sabinene	1.228***	0.145***	0.012	0.012
Δ-3-Carene	7.812***	0.854***	0.038	0.049
α-Phellandrene	5.550***	0.646***	0.054	0.063
Limonene	3.531***	0.437***	0.028	0.029
β-Phellandrene	13.121***	1.376***	0.142	0.163
γ-Terpinene	0.128***	0.024***	0.011	0.012
Terpinolene	1.578***	0.201***	0.012	0.015
Bornylacetate	0.414***	0.065***	0.010	0.010
Total monoterpenes	88.446***	10.149***	0.903	0.975
β-Caryophyllene	0.200***	0.031***	0.007	0.009
α-Humulene	0.144***	0.022**	0.004	0.004
γ-Muurolene	0.422***	0.048**	0.008	0.010
Germacrene	2.898***	0.410***	0.072	0.068
Bicyclogermacrene	0.354***	0.027*	0.005	0.007
Δ-Cadinene	1.181***	0.138***	0.026	0.037
Total sesquiterpenes	5.193***	0.648*	0.110	0.119
Total terpenes	93.639***	10.797***	1.019	1.094

*$P<0.05$, **$P<0.01$; ***$P<0.001$

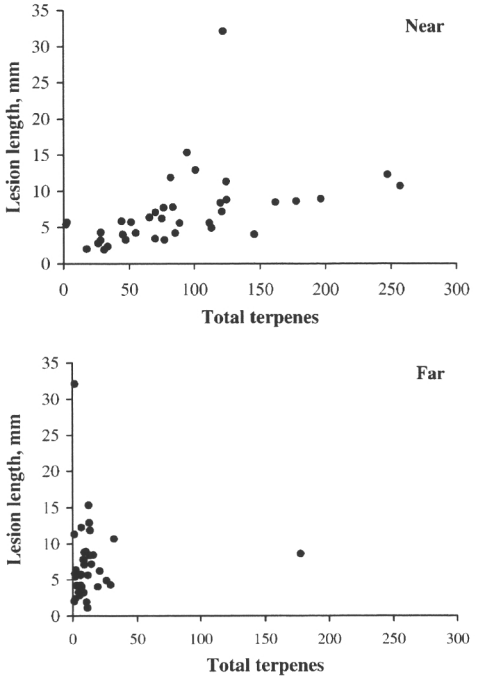

Fig. 5 a Relationship between lesion length and total terpene concentration near the site of fungal inoculation ($r=0.431$, $n=38$, $P<0.01$). **b** The corresponding relationship at the outer border of the lesion was not significant ($r=0.260$, $n=39$, $P>0.01$)

total stilbene glycoside concentration did not correlate with the lesion length near the inoculation site ($r=-0.338$, $n=33$, $P>0.05$) or at the outer border of the reaction lesion ($r=0.066$, $n=39$, $P>0.05$).

On the other hand, the stilbene aglycone isohapontigenin and total aglycones were detected in higher concentrations near the fungal inoculation site than at the other sampling points (Fig. 2). Total aglycone concentration did not correlate with lesion length (data not shown). It correlated positively with the total terpene concentration at the far end of the lesion ($r=0.317$, $n=39$, $P<0.05$) but not with that near the inoculation site ($r=0.237$, $n=38$, $P>0.05$). In N-fertilized trees, near the inoculation site the concentration of resveratrol was significantly lower than in the phloem of unfertilized trees. In addition, the concentration of total stilbene aglycones decreased significantly after N fertilization compared to values for the corresponding unfertilized phloem (Fig. 2).

The detection frequencies of stilbene glycosides near the inoculation site were significantly lower than in mechanically wounded or unwounded phloem (Fig. 3). Correspondingly, near the inoculation site the frequencies of stilbene aglycones were constant. When the detection frequencies of glycosides and aglycones in different fertilization treatments were compared to those of the corresponding unfertilized control, the differences were usually not significant.

Terpenes

The main monoterpenes both in the reaction lesion and in the constitutive phloem tissue were α-pinene, β-pinene, β-phellandrene, Δ-3-carene and α-phellandrene, which made up 82% of the total terpene fraction (Fig. 4).

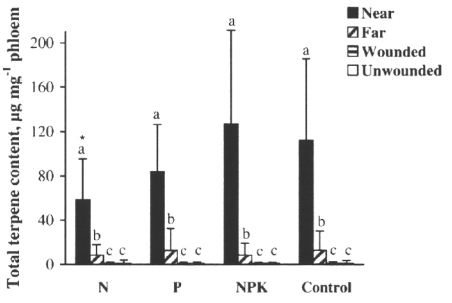

Fig. 6 Total terpene concentrations (µg mg^{-1} fresh phloem, mean±SD) in fungus-inoculated, mechanically wounded and unwounded phloem, grouped according to fertilization. Labels as in Fig. 2

Minor amounts of the monoterpenes limonene, terpinolene and sabinene occurred in all trees. The sesquiterpene fraction was found to consist mainly of germacrene and Δ-cadinene. Each of the other mono- and sesquiterpenes represented <1% of the total terpene fraction.

The average total concentration of terpenes in the phloem near the inoculation site was almost 94 times greater than that of unwounded and mechanically wounded trees (Table 1). In the outer border of the reaction lesion the average terpene concentration was about 11 times greater than the constitutive values. Near the inoculation site the lesion length and the total terpene concentration were positively correlated (Fig. 5a). This was not, however, the case at the outer border of the lesion (Fig. 5b). In all terpenes studied the difference between inoculation treatments was highly significant (Table 1). This difference was most marked in the monoterpenes Δ-3-carene, limonene, terpinolene, sabinene and β-pinene, which near the inoculation site had concentrations >100 times higher than the constitutive values (Table 1). After mechanical wounding, terpene quantity remained stable compared to that in unwounded phloem.

Near the inoculation site, the total terpene concentration of N-fertilized trees was significantly lower than the values for unfertilized control trees; according to the Dunnett-test, the mean difference was –0.59 and P=0.008. The total terpene concentration was about half that found in the control (Fig. 6). Other sampling sites or fertilization treatments did not differ significantly from the corresponding unfertilized control. On the other hand, in some cases the percentage composition of the terpene fraction differed significantly after fertilization treatment (Fig. 4; Table 2). Of the total terpene fraction, the proportion of α-pinene increased after N and P fertilizations, and the proportion of germacrene increased after NPK fertilization. The proportions of β-pinene and β-phellandrene decreased after N and P fertilization.

Table 2 Differences in terpene composition (%) of the total terpene pool between fertilized and unfertilized phloem. Comparisons were made to the unfertilized control with one-way ANOVA. Values are means of all sampling points (Near, Far, Wounded, Unwounded)

	N	P	NPK	Control
Tricyclene	0.34	0.36	0.18	0.27
α-Pinene	34.15***	34.32***	29.48	28.29
Fenchene	0.13			0.10
		0.30***	0.72***	
Camphene			0.64	0.59
	0.80***	0.98***		
β-Pinene	24.71***	24.04***	28.40	27.87
Sabinene	1.12	1.18	1.56	1.26
Δ-3-Carene	5.38	5.90	6.47	6.04
α-Phellandrene	5.54	5.52	4.98	5.42
Limonene	4.31	3.54		3.69
			2.72***	
β-Phellandrene	12.46***	11.35***	14.36	15.13
γ-Terpinene	1.26*	1.23*	0.66*	0.96
Terpinolene	1.53	1.75	1.57	1.56
Bornylacetate			0.68	0.70
	1.30***	1.18***		
Monoterpenes	92.94	91.31	88.82*	91.76
β-Caryophyllene		0.59	0.76	0.59
	0.93***			
α-Humulene	0.23	0.27	0.40	0.27
γ-Muurolene	0.63	0.510*	0.96	0.86
Germacrene	2.980*	4.75		3.78
			8.25***	
Bicyclogermacrene	0.35	0.37	0.68	0.57
Δ-Cadinene	2.23	2.60	2.27	2.28
Sesquiterpenes	7.06	8.69	12.07*	8.24

*P<0.05, **P<0.01, ***P<0.001

Discussion

A mere change in the concentration of allelochemical compounds is not sufficient evidence of changes in resistance to bark beetle attack. Such a change is a general response to stress, wounding or reduced availability of nutrients (Wright et al. 1979; Delorme and Lieutier 1990). Nevertheless, aseptic mechanical wounding generally yields a modest response (Johansson and Stenlid 1985; Lieutier et al. 1989; Raffa and Smalley 1995). In this experiment mechanical wounding did not change the amount of stilbenes and terpenes compared to unwounded phloem. In most cases the bark plugs healed and there were no signs of accumulation of secondary compounds. Mechanical wounding combined with inoculation of sterile malt agar media or autoclaved fungus might have given more variable results, as reported e.g. by Raffa and Smalley (1995).

A low-density method of inoculation was used since the aim was to study phloem chemistry after nutrient levels changed. With this method the tree's defence response is the most efficient mobilizing of all resources against a corresponding attack (Raffa and Berryman 1983). After low-density inoculation, however, lesion length alone is not a good measure of the resistance of the tree or of fungal virulence. Here lesion length was about the same as in a low-density study in Norway spruce after a corresponding incubation time (Krokene and Solheim 1997). In general, small lesions indicate either the weakness of the aggressor or the resistance of the host. The efficient response of a resistant tree might produce a small lesion, while a longer lesion might imply either a physiologically weaker host tree or a more virulent fungus (Krokene and Solheim 1999). The positive relationship between lesion length and total terpene concentration near the inoculation site supports the latter idea. For determining the virulence of fungi, the depth of desiccated sapwood would be a more useful variable than lesion length (Krokene and Solheim 1997, 1999).

In stilbenes, the qualitative changes after inoculation with fungus were more pronounced than the quantitative responses. Concentrations of all stilbene glycosides decreased considerably after inoculation, while the concentration of the aglycone isorhapontigenin increased significantly (Fig. 2). These results agree with those of Woodward and Pearce (1988a), Lindberg et al. (1992) and Brignolas et al. (1995), all of whom observed similar changes a few days after fungal infection. It is known that the β-glycosidase enzymes produced by pathogens, rather than the constitutive host enzymes, are able to metabolize stilbene glycosides to the corresponding aglycones (Johansson and Stenlid 1985; Woodward and Pearce 1988a; Woodward 1992). In this study the increase in β-glycosidase activities in fungus-inoculated phloem might also have been instrumental in the release of aglycones from glycosides (Fig. 2): the longer the reaction lesions were, the less glycosides were present, suggesting that they were actually metabolized. Since β-glycosidase activities were not measured here, we can only speculate about the accumulation of stilbene aglycones after the activities of the β-glycosidase enzymes.

Nevertheless, aglycones cause higher levels of antifungal activity in wounded tissues than glycosides do. For instance, in vitro the antifungal activity of the aglycone isorhapontigenin against the decay fungus *Phaeolus schweinitzii* (Fr.) Pat. was 6 times greater than that of the corresponding glycoside (Woodward and Pearce 1988a). Stilbene aglycones were not observed in previous studies on fungus-inoculated or unwounded phloem of Norway spruce, despite increased stilbene synthase activity (Brignolas et al. 1995, 1998). This may be due to different methods of sample preparation and differences in assay methods, as in a corresponding case in Sitka spruce [*Picea sitchensis* (Bong.) Carr.] (Toscano Underwood and Pearce 1991a). In the present study the accumulation of aglycones was an actual response to fungal inoculation (as shown in Figs. 1, 2) and not an artefact caused by sample preparation.

In healthy spruce, the concentrations of stilbene glycosides are known to differ between trees but may also vary according to age, season, provenance and site (Solhaug 1990; Toscano Underwood and Pearce 1991a, 1991b; Lindberg et al. 1992). With regard to the stilbene glycoside fraction, our results agree with those of Solhaug (1990) and of Lindberg et al. (1992), who concluded that the bark of Norway spruce contains more isorhapontin than astringin. The concentration of stilbene increases towards the outer bark, but in Sitka spruce the procedure for bark-plug sampling still provides an adequate measure of the stilbenes (Toscano Underwood and Pearce 1991a).

The terpene responses of Norway spruce to fungal inoculation were strong and quantitative rather than qualitative. The major terpenes in the reaction lesion did not differ from the constitutive compounds. The enantiomeric composition of monoterpenes, which differs in the different tissues of Norway spruce, and the volatile compounds emitted by the host tree are important in the chemical communication system of the spruce bark beetles and host selection (Ivarsson 1995; Persson et al. 1996). The host tree monoterpene, (-)-α-pinene, acts as a precursor for synthesis of the aggregation pheromone, *cis*-verbenol (Ivarsson 1995). On the other hand, the fungistatic effect of terpenes in conifers seems to be quantitative rather than qualitative; the response of a tree to fungal inoculation is characterized by considerable increases in the concentrations of all the phloem terpenes (Russell and Berryman 1976; Raffa and Berryman 1982a, 1982b; Lieutier et al. 1989; Delorme and Lieutier 1990; Raffa and Smalley 1995). On the basis of our sampling procedure, it is difficult to determine whether terpenoids accumulated here in large quantities after fungal inoculation originated from translocation or from on-site de novo biosynthesis (Lewinsohn et al. 1993). In Norway spruce, parenchyma cells near the fungal inoculation site differentiate into resin ducts (Franceshi et al. 2000; Nagy et al. 2000), which makes long-range translocation of terpenes less obvious. In addition, changes in terpene composition after inoculation support the idea that the neosynthesis occurred around the inoculation site.

Sesquiterpenes and resin acids (Björkman et al. 1991), which occur in small quantities, may not be representa-

tive indicators for testing the CNB hypothesis. Furthermore, resins that require not only the synthesis but also the production of specialized storage structures or compartments may begin to decline sooner than compounds, such as phenolics, that can be sequestered simply by the surrounding cell walls or vacuoles (Lerdau et al. 1994). Previous investigations have also indicated the opposite; C may not be the limiting factor for resin accumulation, but rather physiological and anatomical constraints (Björkman et al. 1991; Muzika 1993; Kytö et al. 1999).

Stilbene compounds that are present in low concentrations after dynamic reactions are difficult to detect, and therefore quantitative changes following changes in resource availability are difficult to observe and document reliably. Trade-off between growth and defence seems to be feasible only when there is actual biosynthetic competition for the same precursor, as in the case of the phenylpropanoids. The CNB hypothesis is not applicable in all cases because not all C-based secondary compounds seem to respond to fertilization in the same way (Lawler et al. 1997; Koricheva et al. 1998, and references therein; Keinänen et al. 1999). For example, total phenolics, which was previously a widely used variable, may include ecologically interesting but opposite responses of secondary compounds, since tannins and non-tannin phenolics have not been separated and identified. Here, for stilbene aglycones and stilbene glycosides, the trends detected in response to fertilization were opposite.

In this study, the accumulation of stilbene aglycones and terpenes was significantly reduced near the inoculation site only after N fertilization, but not by P or NPK fertilizations. Thus, N fertilization might affect the ability of spruce to defend itself against aggression by *C. polonica*. Responses after N fertilization were consistent with the CNB hypothesis. Even so, according to a wide meta-analysis (Koricheva et al. 1998, and references therein), the ability of the CNB hypothesis to predict changes in plant terpenoids in response to experimental manipulations seems weak. That analysis, however, contained many studies in which constitutively occurring secondary compounds were included or changes outside the active growing phase were analysed. The predictions of plant defence theories might best be fulfilled during those times of the year when growth and defence are competing for resources, so that an inverse relationship between N availability and allocation to mobile C-based defences might be expected (Lerdau et al. 1995). Minor changes in growth and defence caused by P or NPK fertilization or by other stress treatments can easily be hidden within natural variation, especially if these changes occur outside the active growing season. Since here sampling was done only once, the lack of reference samples at the beginning of the experiment is a weakness and should be taken into account when results are interpreted. The shift from stilbene glycosides to aglycones in the lesion (Fig. 2) indicates, however, the dynamics involved in the wound reaction.

Here, NPK fertilization increased the total amount of terpenes but decreased the total concentration of stilbene aglycones near the inoculation site. The N-fertilizer in our NPK regime was moderate (144 kg ha^{-1} $year^{-1}$), since the goal was to imitate actual fertilization in practical forestry and not to create artificial responses with overdoses. These results agree with a those of Muzika et al. (1989) and Lerdau et al. (1995), who found that treatment with a high level of N often produced less terpenes, whereas an intermediate level of N apparently had little influence on terpene production. Most likely, our NPK fertilization was too modest to cause proper responses in allocation of secondary metabolites. It might be that NPK fertilization has a more balanced effect on secondary metabolite production than pure N fertilization does. In boreal coniferous forests, N is commonly a growth-limiting factor, whereas P seldom reduces growth; when it does, this typically occurs on peat lands. Thus it is not surprising that P fertilization had no significant effect on secondary metabolite concentrations in this study or in the meta-analysis (Koricheva et al. 1998). This may also be the reason why we found a significant decrease in total terpenes only in the induced response of N-fertilized trees.

In summary, N fertilization resulted in lower concentrations of total terpenes and stilbene aglycones, but P fertilization led to only a minor decrease in the concentration of total terpenes. The CNB hypothesis predicts changes in these groups of secondary metabolites in response to the above-mentioned nutrient changes. On the other hand, the concentrations of stilbene glycosides did not correspond to the predictions of the CNB hypothesis; their role as induced defensive compounds may be less important. However, the effect of fertilization was seen only at the point where the induced wound response was the most extensive, near the inoculation site. Results indicate that further studies of plant-herbivore interactions are needed, in particular, studies using a model in which allocation varies in response to phenological and herbivory demands.

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References

Berryman AA (1969) Responses of *Abies grandis* to attack by *Scolytus ventralis* (Coleoptera: Scolytidae). Can Entomol 101: 1033–1041

Berryman AA (1972) Resistance of conifers to invasion by bark beetle-fungus associations. BioScience 22:598–602

Björkman C, Larsson S, Gref R (1991) Effects of nitrogen fertilization on pine needle chemistry and sawfly performance. Oecologia 86:202–209

Bridges JR (1987) Effects of terpenoid compounds on growth of symbiotic fungi associated with the southern pine beetle. Phytopathology 77:83–85

Brignolas F, Lacroix B, Lieutier F, Sauvard D, Drouet A, Claudot A-C, Yart A, Berryman AA, Christiansen E (1995) Induced responses in phenolic metabolism in two Norway spruce clones after wounding and inoculations with *Ophiostoma polonicum*, a bark beetle-associated fungus. Plant Physiol 109:821–827

Brignolas F, Lieutier F, Sauvard D, Christiansen E, Berryman AA (1998) Phenolic predictors for Norway spruce resistance to the bark beetle *Ips typographus* (Coleoptera: Scolytidae) and an associated fungus, *Ceratocystis polonica*. Can J For Res 28: 720–728

Bryant JP, Chapin FS III, Klein DR (1983) Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. Oikos 40:357–368

Chapin FS III (1980) The mineral nutrition of wild plants. Annu Rev Ecol Syst 11:233–260

Christiansen E (1985) *Ips/Ceratocystis*-infection of Norway spruce: what is a deadly dosage? Z Angew Entomol 99:6–11

Cobb FW Jr, Krstic M, Zavarin E, Barber HW Jr (1968) Inhibitory effects of volatile oleoresin components on *Fomes annosus* and four *Ceratocystis* species. Phytopathology 58:1327–1335

Delorme L, Lieutier F (1990) Monoterpene composition of the preformed and induced resins of Scots pine, and their effect on bark beetles and associated fungi. Eur J For Pathol 20: 304–316

Franceschi R, Krekling T, Berryman AA, Christiansen E (1998) Specialized phloem parenchyma cells in Norway spruce (Pinaceae) bark are an important site of defense reactions. Am J Bot 85:601–615

Franceschi VR, Krokene P, Krekling T, Christiansen E (2000) Phloem parenchyma cells are involved in local and distant defense responses to fungal inoculation or bark-beetle attack in Norway spruce (Pinaceae). Am J Bot 87:314–326

Gershenzon J (1994) Metabolic costs of terpenoid accumulation in higher plants. J Chem Ecol 20:1281–1328

Hart JH (1981) Role of phytostilbenes in decay and disease resistance. Annu Rev Phytopathol 19:437–458

Herms DA, Mattson WJ (1992) The dilemma of plants: to grow or defend. Quart Rev Biol 67:283–335

Hurlbert SH (1984) Pseudoreplication and the design of ecological field experiments. Ecol Monogr 54:187–211

Ivarsson P (1995) The pheromone systems of the spruce bark beetles, *Ips duplicatus* and *I. typographus*: biosynthesis and regulation. Dissertation. Faculty of Natural Sciences, Göteborg University, Göteborg

Jeffers JNR (1960) Experimental design and analysis in forest research. Almqvist and Wiksell, Stockholm

Johansson M, Stenlid J (1985) Infection of roots of Norway spruce (*Picea abies*) by *Heterobasidion annosum*. 1. Initial reactions in sapwood by wounding and infection. Eur J For Pathol 15: 32–45

Keinänen M, Julkunen-Tiitto R, Mutikainen P, Walls M, Ovaska J, Vapaavuori E (1999) Trade-offs in phenolic metabolism of silver birch: effects of fertilization, defoliation, and genotype. Ecology 80:1970–1986

Klepzig KD, Kruger EL, Smalley EB, Raffa KF (1995) Effects of biotic and abiotic stress on induced accumulation of terpenes and phenolics in red pines inoculated with bark beetle-vectored fungus. J Chem Ecol 21:601–626

Koricheva J, Larsson S, Haukioja E, Keinänen M (1998) Regulation of woody plant secondary metabolism by resource availability: hypothesis testing by means of meta-analysis. Oikos 83:212–226

Krokene P, Solheim H (1996) Fungal associates of five bark beetle species colonizing Norway spruce. Can J For Res 26:2115–2122

Krokene P, Solheim H (1997) Growth of four bark-beetle-associated blue-stain fungi in relation to the induced wound response in Norway spruce. Can J Bot 75:618–625

Krokene P, Solheim H (1999) What do low-density inoculations with fungus tell us about fungal virulence and tree resistance? In: Lieutier F, Mattson WJ, Wagner MR (eds) Physiology and genetics of tree-phytophage interactions. INRA Editions, Paris, pp 353–362

Kytö M, Niemelä P, Annila E, Varama M (1999) Effects of forest fertilization on the radial growth and resin exudation of insect-defoliated Scots pines. J Appl Ecol 36:763–769

Lawler IR, Foley WJ, Woodrow IE, Cork SJ (1997) The effects of elevated CO_2 atmospheres on the nutritional quality of *Eucalyptus* foliage and its interaction with soil nutrient and light availability. Oecologia 109:59–68

Lerdau M, Litvak M, Monson R (1994) Plant chemical defense: monoterpenes and the growth-differentiation balance hypothesis. Trends Ecol Evol 9:58–61

Lerdau M, Matson P, Fall R, Monson R (1995) Ecological controls over monoterpene emissions from Douglas-fir (*Pseudotsuga menziesii*). Ecology 76:2640–2647

Lewinsohn E, Gijzen M, Muzika RM, Barton K, Croteau R (1993) Oleoresinosis in grand fir (*Abies grandis*) saplings and mature trees. Plant Physiol 101:1021–1028

Lieutier F, Cheniclet C, Garcia J (1989) Comparison of the defense reactions of *Pinus pinaster* and *Pinus sylvestris* to attacks by two bark beetles (Coleoptera: Scolytidae) and their associated fungi. Environ Entomol 18:228–234

Lindberg M, Lundgren L, Gref R, Johansson M (1992) Stilbenes and resin acids in relation to the penetration of *Heterobasidion annosum* through the bark of *Picea abies*. Eur J For Pathol 22:95–106

Muzika RM (1993) Terpenes and phenolics in response to nitrogen fertilization: a test of the carbon/nutrient balance hypothesis. Chemoecology 4:3–7

Muzika RM, Pregitzer KS, Hanover JW (1989) Changes in terpene production following nitrogen fertilization of grand fir (*Abies grandis* (Dougl.) Lindl.) seedlings. Oecologia 80:485–489

Myers JL, Well AD (1995) Research design and statistical analysis. Erlbaum, Hillsdale, N.Y.

Nagy NE, Franceschi VR, Solheim H, Krekling T, Christiansen E (2000) Wound-induced traumatic resin duct development in stems of Norway spruce (Pinaceae): anatomy and cytochemical traits. Am J Bot 87:302–313

Nicholson RL, Hammerschmidt R (1992) Phenolic compounds and their role in disease resistance. Annu Rev Phytopathol 30: 369–389

Persson M, Sjödin K, Borg-Karlson A-K, Norin T, Ekberg I (1996) Relative amounts and enantiomeric compositions of monoterpene hydrocarbons in xylem and needles of *Picea abies*. Phytochemistry 42:1289–1297

Pohjola J (1993) A Headspace gas chromatographic study on the variation of needle volatile terpenes in Scots pine (*Pinus sylvestris* L.). Dissertation. Pharmacognosy Division, Department of Pharmacy, University of Helsinki, Helsinki

Raffa KF, Berryman AA (1982a) Accumulation of monoterpenes and associated volatiles following inoculation of grand fir with a fungus transmitted by the fir engraver, *Scolytus ventralis* (Coleoptera: Scolytidae). Can Entomol 114:797–810

Raffa KF, Berryman AA (1982b) Physiological differences between lodgepole pines resistant and susceptible to the mountain pine beetle and associated microorganisms. Environ Entomol 11:486–492

Raffa KF, Berryman AA (1983) Physiological aspects of lodgepole pine wound responses to a fungal symbiont of the mountain pine beetle, *Denroctonus ponderosae* (Coleoptera: Scolytidae). Can Entomol 115:723–734

Raffa KF, Smalley EB (1995) Interaction of pre-attack and induced monoterpene concentrations in host conifer defense against bark beetle-fungal complexes. Oecologia 102:285–295

Raffa KF, Berryman AA, Simasko J, Teal W, Wong BL (1985) Effects of grand fir monoterpenes on the fir engraver, *Scolytus ventralis* (Coleoptera: Scolytidae), and its symbiotic fungus. Environ Entomol 14:552–556

Reid RW, Whitney HS, Watson JA (1967) Reactions of lodgepole pine to attack by *Dendroctonus ponderosae* Hopkins and blue stain fungi. Can J Bot 45:1115–1126

Russell CE, Berryman AA (1976) Host resistance to the fir engraver beetle. 1. Monoterpene composition of *Abies grandis* pitch blisters and fungus-infected wounds. Can J Bot 54:14–18

Schultz TP, Boldin WD, Fisher TH, Nicholas DD, McMurtrey KD, Pobanz K (1992) Structure-fungicidal properties of some 3- and 4-hydroxylated stilbenes and bibenzyl analogues. Phytochemistry 31:3801–3806

Snedecor GW, Cochran WG (1980) Statistical methods. Iowa State University Press, Ames, Iowa

Solhaug KA (1990) Stilbene glucosides in bark and needles from *Picea* species. Scand J For Res 5:59–67

Solheim H (1988) Pathogenicity of some *Ips typographus*-associated blue-stain fungi to Norway spruce. Commun Norw For Res Inst 40:1–11

Toscano Underwood CD, Pearce RB (1991a) Astringin and isorhapontin distribution in Sitka spruce trees. Phytochemistry 30: 2183–2189

Toscano Underwood CD, Pearce RB (1991b) Variation in the levels of the antifungal stilbene glucosides astringin and isorhapontin in the bark of Sitka spruce [*Picea sitchensis* (Bong.) Carr.]. Eur J For Pathol 21:279–289

Wingfield MJ, Seifert KA, Webber JF (1993) *Ceratocystis* and *Ophiostoma*. Taxonomy, ecology and pathogenicity. APS, St. Paul, Minn.

Woodward S (1992) Responses of gymnosperm bark tissues to fungal infections. In: Blanchette RA, Biggs AR (eds) Defense mechanisms of woody plants against fungi. Springer, Berlin Heidelberg New York, pp 62–75

Woodward S, Pearce RB (1988a) The role of stilbenes in resistance of Sitka spruce [*Picea sitchensis* (Bong.) Carr.] to entry of fungal pathogens. Physiol Mol Plant Pathol 33:127–149

Woodward S, Pearce RB (1988b) Wound-associated responses in Sitka spruce root bark challenged with *Phaeolus schweinitzii*. Physiol Mol Plant Pathol 33:151–162

Wright LC, Berryman AA, Gurusiddaiah S (1979) Host resistance to the fir engraver beetle, *Scolytus ventralis* (Coleoptera: Scolytidae). 4. Effect of defoliation on wound monoterpene and inner bark carbohydrate concentrations. Can Entomol 111: 1255–1262

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ORIGINAL ARTICLE

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Soluble carbohydrates, radial growth and vigour of fertilized Norway spruce after inoculation with blue-stain fungus, *Ceratocystis polonica*

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Abstract The aim of this study was to determine whether fertilization and the consequent increase in growth reduce the allocation of soluble carbohydrates in response to an induced wound. Norway spruce trees fertilized with N, P or NPK were artificially infected with Ceratocystis polonica, a blue-stain fungus associated with the spruce bark beetle Ips typographus. N and NPK fertilization treatments increased radial growth of the stem and the vigour indices. The concentration of total soluble carbohydrates in the outer border of the lesion was significantly decreased in P-fertilized trees compared to corresponding unfertilized trees. However, changes in the soluble carbohydrate concentration caused by fungal inoculation were more pronounced than changes caused by fertilization. The main soluble carbohydrate was sucrose, and after fungal inoculation its concentration decreased considerably near the site of inoculation. Thus, near the site of fungal inoculation the concentration of total soluble carbohydrates also decreased significantly compared to corresponding values in unwounded phloem. Despite the fact that in all fertilized trees the radial growth of the stem increased, the only indication that enhanced growth might reduce the level of resistance was the modest positive correlation between lesion length and radial growth of the stem.

Keywords Soluble sugars · Sugar alcohols · Carbon/nutrient balance hypothesis · Resource allocation · Vigour index

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Introduction

Bark beetle-fungus attack causes accumulation of secondary metabolites in the phloem tissues of conifers. In response to attack, the parenchyma cells in the phloem produce large quantities of C-based secondary compounds (CBSCs) that, compared to primary metabolites, are energetically costly to synthesize (Lorio 1986; Gershenzon 1994a, 1994b). The attack leads to a decrease in the amount of soluble carbohydrates in the inner bark and sapwood (Shrimpton 1973; Wright et al. 1979; Christiansen and Ericsson 1986; Cook and Hain 1987). Furthermore, primary fungal invaders of the phloem utilize sugars as nutrients. Growth of the fungus is first delayed by the removal of essential nutrients, like glucose and fructose, and later by secondary metabolites, which reduce the availability of water and oxygen in the lesion (Wong and Berryman 1977; Raffa and Berryman 1982). Finally, traumatic formation of resin ducts and wound periderm seal the lesion (Reid et al. 1967; Franceschi et al. 2000; Nagy et al. 2000).

Carbohydrates are especially important because they are direct products of photosynthesis and are therefore the primary energy-storage compounds and the basic organic substances from which most other organic compounds found in plants are synthesized (Kozlowski and Pallardy 1997). The ability of trees to withstand attack by bark beetles and their associated fungi is thought to be linked to the amount of carbohydrates that can be utilized for defence reactions. Thus, the ability of a tree to resist attacks by bark beetles is directly related to its physiological status and vigour (Mulock and Christiansen 1986; Christiansen et al. 1987; Paine et al. 1997). Therefore, any environmental factor that restricts the size of the canopy or its photosynthetic efficiency can weaken the resistance of the tree. Production of carbohydrates in plant tissues, if this exceeds the amount required for growth, favours the synthesis of C-based secondary metabolites, e.g. phenolics and terpenes (Bryant et al. 1983; Herms and Mattson 1992). The quantitative ability of a tree to respond to infection in-

creases until the tree reaches maturity, and then declines with age (Raffa and Berryman 1982). In plants, in general, the biosynthesis of terpenoids proceeds via two independent pathways: first, sesquiterpenes, triterpenes and sterols are biosynthesized via the cytosolic, classic acetate/mevalonate pathway or via the alternative, non-mevalonate 1-deoxy-D-xylulose-5-phosphate pathway for the biosynthesis of plastidic terpenoids, such as mono- and diterpenes (Lichtenthaler 1999). Both pathways form the active C_5-unit isopentenyl diphosphate as the precursor from which all other terpenoids are formed via head-to-tail addition (Lichtenthaler 1999). Phenolics are derived from carbohydrates via the shikimate pathway through which about one fifth of all the C fixed by plants flows (Matsuki 1996). According to the C/nutrient balance (CNB) hypothesis, fertilization with a growth-limiting nutrient or release of some other nutrient will stimulate growth more strongly than photosynthesis (Chapin 1980), so that concentrations of carbohydrates and C-based secondary metabolites decline (Bryant et al. 1983; Herms and Mattson 1992).

The main aim of the present study was to investigate interactions between soluble carbohydrates, defence mechanisms and radial growth of the stem of Norway spruce. The specific objectives were:

1. To examine the effect of artificial inoculation with the blue-stain fungus *Ceratocystis polonica* Siem. Moreau, transmitted by the spruce bark beetle, *Ips typographus* L., on the composition of soluble carbohydrates in the phloem.
2. To determine whether fertilization affects the relative amounts of soluble carbohydrates.
3. To examine the interactions between soluble carbohydrates, terpenes, stilbenes, radial growth of the stem in Norway spruce and the length of lesions caused by *C. polonica*.

A companion paper (Viiri et al. 2001) indicated that after fungal inoculation in Norway spruce the total amount of terpenes increased and the total concentrations of stilbene glycoside decreased. On the other hand, N fertilization decreased the concentrations of both total terpenes and total stilbene aglycones (Viiri et al. 2001).

Material and methods

Experimental area and fungal inoculations of trees

The study area was located in a mature natural stand of Norway spruce [*Picea abies* (L.) Karst.] in Karttavuori, Vesijako, southern Finland (see Viiri et al. 2001 for details). At the end of the experiment, the experimental trees averaged 34±0.5 cm in diameter at breast height and 28±0.2 m in total height. Four plots of 0.5 ha were marked, and fertilization treatments [N (173 kg ha^{-1} year^{-1}; NH_4-N 13.5%; NO_3-N 13.5%; Ca 3%; Mg 1%; S 3%; B 0.02%), P (41 kg ha^{-1} year^{-1}; total P 9%; P water soluble 8%; Ca 20%; S 11%) and NPK (800 kg ha^{-1} year^{-1}; NH_4-N 11%; NO_3-N 7%; total P 5%; P water soluble 3.6%; K 10%; Ca 3%; Mg 0.5%; S 3%; B 0.02%; Se 0.001%)] were spread by hand on 4 May 1993. The control treatment was a non-fertilized plot. Due to limited space, we used a single plot for each fertilization treatment and one control plot. Thus, our experimental design was pseudoreplicated (Hurlbert 1984); this is taken into account in the interpretation of results, where fertilization treatments are compared with the control, not with each other. From each plot, 30 trees that were free of visible wounds, a total of 120 trees, were selected. Two trees that were infected with root-rot and two attacked by *I. typographus* were omitted from the analysis.

In June 1994 a culture of blue-stain fungus, *C. polonica*, on malt extract agar in Petri dishes was inoculated into ten trees per fertilization treatment. The fungus was inoculated into the phloem with a cork borer (diameter 5 mm), and the bark plug was re-inserted into the hole according to the method originally described by Wright (1933). Each tree received four inoculations, one at each of the cardinal points of the compass, 1.3 m above ground level. For mechanical wounding, ten trees per fertilization treatment were injured with a cork-borer in the same way as for inoculation, but without the fungus and malt agar. In the middle of August, after a 2-month incubation period, phloem samples were taken and the vertical length of each lesion was measured. Around each site of fungal inoculation, four phloem samples were taken with the cork-borer (diameter 15 mm) to the level of the cambium: two from the distal ends of the visible reaction lesion (henceforth called "far" samples) and two immediately above and below the site of inoculation (henceforth called "near" samples). Two samples were taken from around the site of mechanical wounding, one above the wounding site and the other below the wounding site. In cases where there was visible lesion formation around the mechanical wounding (necrotic area more than 3 mm at the upper and lower side of the inoculation site, n=11), these necrotic tissues were included as part of the samples. Two samples of phloem per tree from intact trees were used as unwounded controls. Phloem samples of intact trees were taken at the same height and location as in mechanically wounded and fungus-inoculated trees. All samples were immediately placed on dry ice and stored at –20°C until analysed.

Tree characteristics

At the end of the growing season, on 5 October 1994, the experimental trees were felled and their main characteristics were measured: age, height, diameter at breast height, crown length, bark and phloem thicknesses (Table 1). Stem discs were cut from each tree at a height of 6.1 m for assessment of radial growth of the stem during the last 5 years (1990–1994) and determination of the tree-vigour index (Waring et al. 1980; Münster-Swendsen 1987). The cross-sectional area of the sapwood is rather constant from breast height to the base of the live crown. The vigour indices were calculated as the ratio of the cross-sectional area of the annual ring and the basal area of the sapwood. Diameters were measured twice in opposite directions, and all other measurements of thickness (annual rings, bark, phloem and sapwood) were means of four measurements made at four positions along the radii 90° apart. In some cases the heartwood-sapwood border was impossible to distinguish precisely due to the changing direction of the tracheids at the base of the branch whorls or similarity in the natural colours of the sap and heartwood. In these cases (n=30), the thickness of the sapwood was estimated from the regression model y=13.111+0.655x (r^2=0.183, n=90) based on the actual diameter (x) and sapwood (y) measurements in the same experiment.

Analysis of chemical elements

When trees were felled at the end of the experiment, needle samples for analyses of chemical elements were taken from the mid crown on the southern side of the trees. From all experimental trees, the needles of the current year class, 1994, and the previous year class, 1993, were sampled separately. After all sampled needles had been dried for 24 h at 60°C and ground, the total concentrations of C and N were determined with a CHN elemental analyser (CHN-1000; Leco). The concentrations of other elements

Table 1 Characteristics (mean±SD) of the experimental trees at the end of the experiment

	Fertilization treatments			
	N	P	NPK	Control
Height (m)	27.8±2.4	29.2±1.9	28.3±2.3	28.0±2.6
DBH (cm)	33.6±4.7	33.7±4.1	35.0±6.3	32.3±4.5
Live crown ratio (%)	41.6±9.8	44.6±8.8	31.0±9.4***	43.1±8.1
Age (years)	80±5	83±6	81±6	79±8
Bark thickness (mm)	2.60±0.6	2.67±0.5	2.24±0.7	2.39±0.7
Phloem thickness (mm)	0.35±0.07	0.34±0.06	0.33±0.06	0.32±0.07
Sapwood thickness (cm)	3.12±0.72	3.23±0.63	3.60±0.61*	3.08±0.59
n	29	30	29	28

*$P<0.05$, ***$P<0.001$ in relation to the corresponding unfertilized control

were determined by using an inductively coupled plasma atomic emission spectrometer (ARL-3580) after dry ashing and extraction with HCl from separate needle samples.

Extraction and analysis of soluble carbohydrates

Soluble carbohydrates were extracted according to the method of Mason and Slover (1971). The phloem samples were ground in liquid N, and 100 mg of ground phloem powder was then extracted with 2 ml of 80% v/v EtOH. The ethanol contained 1,000 ppm phenyl-β-D-glucoside (Sigma) as internal standard (Marcy and Carroll 1982). The extraction liquid was placed in an ultrasonic bath for 45 min, after which it was left overnight to stabilize. The extract was shaken on an orbital shaker and then centrifuged for 20 min at 4,332 *g*; 500 µl of supernatant was evaporated to complete dryness under a flow of N. After all solvent had evaporated, the samples were silylated for 2 h at room temperature with 400 µl trimethylsilyl-imidazole (TMSI) reagent prepared from 100 ml dry pyridine and 21 ml TMSI (Fluka) and preserved in complete dryness over silica gel. The silylated samples were shaken on an orbital shaker and preserved at –20°C until analysed. The external carbohydrate standard was prepared by dissolving each carbohydrate separately [50 mg fructose, 100 mg glucose, 50 mg sorbitol, 50 mg myo-inositol and 50 mg sucrose (Merck)] in 25 ml of 80% EtOH.

TMSI derivatives of soluble carbohydrates were analysed with a Hewlett-Packard 5890 gas chromatograph with a HP-5 (Hewlett-Packard) capillary column (25 m×0.2 mm×0.3 µm) equipped with a mass spectrometry (Hewlett-Packard 5988A) system. The initial temperature was 110°C, increasing at a rate of 10°C min^{-1} to a final temperature of 300°C. Split injection was used, and the injection temperature was 260°C. The split flow was 20 ml min^{-1} and the carrier gas was helium. Soluble carbohydrates were quantified and identified by comparing retention times and mass spectra to those of the carbohydrate standards. The results are expressed as microgram of soluble carbohydrates per milligram of fresh phloem tissue. Detailed methods and results for stilbene and terpene analysis are presented in a previous paper (Viiri et al. 2001).

Statistical analysis

Univariate ANOVA was used to examine overall differences between inoculation methods. The means of chemical compounds calculated from opposite sides of the inoculation site were used in the analysis. For mechanically wounded trees and unwounded controls, the means of two sampling sites were used. Homogeneity of the variances was tested with Levene's test (Snedecor and Cochran 1980). The Dunnett test (Myers and Well 1995) was used to examine differences between fertilized plots and the unfertilized control and differences within inoculation treatments. If the assumption of homogeneity was not fulfilled (Jeffers 1960), nonparametric Dunnett *C*-tests were used. The values presented in the tables and figures are untransformed means, and if not mentioned otherwise, the level of significance is 5%. Tree-wise means of variables were calculated for correlation analysis. Concentrations of each individual soluble carbohydrate showed a significant positive correlation with total soluble carbohydrate concentration at both sampling points in fungus-inoculated trees (data not shown). The trend was the same in mechanically wounded and unwounded treatments. Therefore, to simplify the presentation of correlation analyses, the total soluble carbohydrate concentrations were used. Spearman's rho was used as the correlation coefficient for examining relationships between total soluble carbohydrates, total terpenes, total stilbene glycosides, total stilbene aglycones, lesion length and radial growth of the stem in experimental trees.

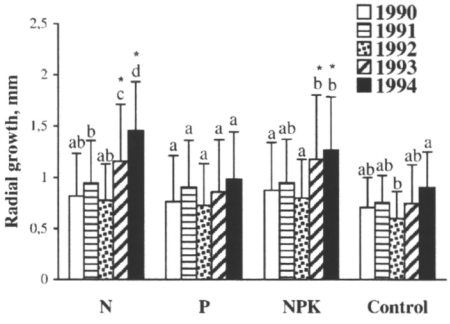

Fig. 1 Radial growth of the stem (mean±SD) of experimental trees before (*1990–1992*) and after (*1993, 1994*) fertilization, grouped according to fertilization treatments. The *asterisk* indicates a significant difference from the unfertilized control trees at the 5% level. *Within a treatment*, bars with *different letters* differ significantly from each other at the 5% level. *n* in fertilization treatments: N=29, P=30, NPK=29, Control=28

Results

Radial growth and vigour of trees

After the experiment, tree characteristics such as age, height and diameter at breast height did not differ significantly between fertilized and unfertilized trees. In trees fertilized with NPK the crown ratio was significantly lower and the sapwood thickness significantly greater than in unfertilized trees (Table 1). All trees, including unfertilized controls, had greater radial growth of the stem in 1994 than they had before fertilization (Fig. 1). Before fertilization, there were no significant differences in the radial growth of the stem. Due to fertilization, the radial growth of the stem in the experimental trees increased in the year when fertilizers were applied and even more dur-

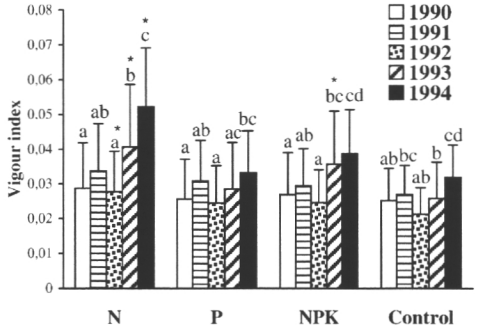

Fig. 2 Vigour indices (mean±SD) of experimental trees before (*1990–1992*) and after (*1993, 1994*) fertilization, grouped according to fertilization treatments. The *asterisk* indicates a significant difference from the unfertilized control trees at the 5% level. *Within a treatment*, bars with *different letters* differ significantly from each other at the 5% level

ing the following year. In the 2 years after fertilization, the growth of the annual ring in N- and NPK-fertilized trees was significantly greater than in unfertilized trees.

Vigour indices in all trees were higher in 1994 than in 1993 (Fig. 2). In both years, the vigour indices were significantly higher in N-fertilized trees than in the unfertilized controls. In 1993, the vigour index was significantly higher in NPK-fertilized trees than in the control, but in 1994 the difference was not significant. When all fertilization treatments were pooled, vigour indices correlated positively with total concentration of soluble carbohydrate only in the mechanically wounded trees (data not shown).

Nutrients and soluble carbohydrates

N and NPK fertilizers increased the concentration of foliar N up to the level recommended for spruce on this

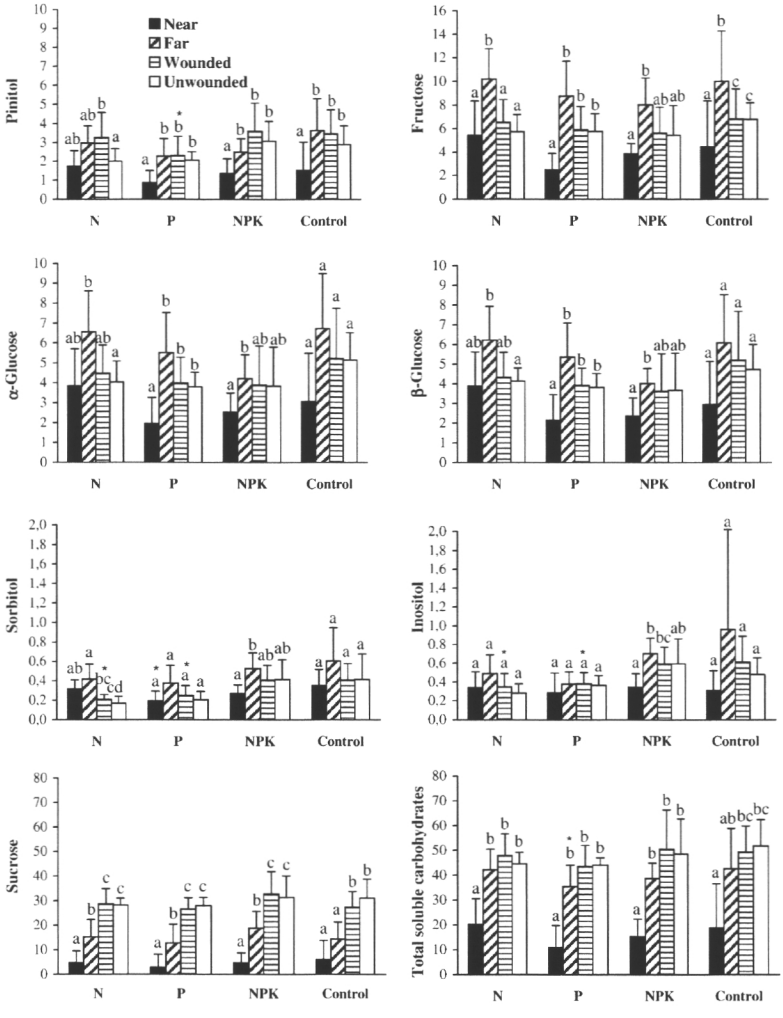

Fig. 3 Carbohydrate concentrations (μg mg^{-1} fresh phloem, mean±SD) in Norway spruce phloem inoculated with *Ceratocystis polonica* near the inoculation site (*near*, e.g. middle part of the lesion), at the outer border of the lesion (*far*), mechanically wounded (*wounded*) and unwounded samples (*unwounded*) grouped according to fertilization. The *asterisk* indicates a significant difference from the unfertilized control trees at the 5% level. *Within a treatment*, bars with *different letters* differ significantly from each other at the 5% level

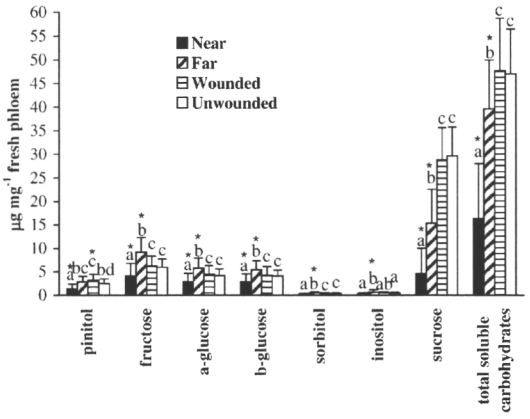

Fig. 4 Carbohydrate concentrations (µg mg^{-1} fresh phloem, mean±SD) in Norway spruce phloem inoculated with *C. polonica* grouped according to sampling site: near (n=38); far (n=39); wounded (n=38); and unwounded samples (n=39). Each fertilization treatment and the control were combined. Values indicated with an *asterisk* at the end of a bar differ significantly from unwounded phloem at the 5% level. For the *same compound*, bars labelled with a *different letter* differ significantly from each other at the 5% level. For sampling site, see Fig. 3

Table 2 Concentrations of chemical elements (% of dry weight) and C/N ratios of the 1993 and 1994 needle classes at the end of the experiment on 5 October 1994. Fertilization treatments were applied on 4 May 1993

	Needle class	Fertilization treatments			
		N	P	NPK	Control
N (mg g^{-1})	1993	16.59***	10.82	14.99***	10.76
	1994	16.97***	13.17	17.21***	13.01
P (mg g^{-1})	1993	1.05	2.02***	1.28***	1.03
	1994	1.67	2.43***	2.09***	1.58
K (mg g^{-1})	1993	4.48	4.84	4.60	4.55
	1994	8.04*	7.22	7.92*	7.01
Mg (mg g^{-1})	1993	0.92***	1.12	0.93***	1.21
	1994	0.94***	1.28	0.92***	1.27
Mn (mg g^{-1})	1993	1.02	1.26	0.94*	1.14
	1994	0.58***	0.89*	0.52***	0.75
Ca (mg g^{-1})	1993	5.30	5.41	5.67	5.74
	1994	2.63***	3.65	2.69***	3.56
B (µg g^{-1})	1993	11.35*	12.33	14.61	14.30
	1994	11.34	12.07	12.59	12.33
C/N ratio	1993	34.6***	51.6	37.6***	51.6
	1994	33.4***	42.5	32.4***	42.9
n		29	30	28	29

*P<0.05, ***P<0.001 in relation to the corresponding unfertilized control

Table 3 Lesion lengths caused by *Ceratocystis polonica* (mean±SD) and Spearman's rho non-parametric correlations of lesion length with radial growth of the stem and the vigour index. Tree-wise means of four inoculations per tree

	N	P	NPK	Control	All trees
Lesion length (cm)	5.4±4.0	9.0±8.5	6.5±3.8	7.5±3.0	7.1±5.3
Radial growth 1990	0.32	0.80**	0.61	0.48	0.53**
Radial growth 1991	0.56	0.45	0.57	0.15	0.38*
Radial growth 1992	0.54	0.74*	0.56	0.26	0.46**
Radial growth 1993	0.61	0.77**	0.65*	0.32	0.42**
Radial growth 1994	0.51	0.51	0.58	0.38	0.30
Vigour index 1990	0.39	0.52	0.48	0.46	0.18
Vigour index 1991	0.21	0.16	0.49	0.04	0.30
Vigour index 1992	0.33	0.59	0.63	0.25	0.34*
Vigour index 1993	0.27	0.58	0.67*	0.29	0.19
Vigour index 1994	0.20	0.38	0.60	0.40	0.40*
n	10	10	10	9	39

*P<0.05, **P<0.01

type of site (Jukka 1988) (Table 2). In 1994 the Ca and Mg concentrations of the needles were significantly lower in N- and NPK-fertilized trees, despite the fact that both N fertilization treatments contained Ca and Mg. In 1994 the Mn concentrations of the needles were also significantly lower in N- and NPK-fertilized trees.

The main soluble carbohydrate was sucrose, which alone made up most of the total amount of soluble carbohydrates (Fig. 3). Pinitol, fructose, α-glucose, β-glucose and small amounts of sorbitol and inositol were also identified. The phloem near the inoculation site contained smaller amounts of total soluble carbohydrates, pinitol, fructose, glucose and sucrose, than the unwounded phloem did (Fig. 3). On the other hand, the concentrations of pinitol, fructose, α-glucose, β-glucose, sorbitol and inositol in the outer border of the lesion were higher than in the unwounded phloem.

In the fertilization treatments, the concentration of total soluble carbohydrates was significantly lower in the outer border of the lesion only in P-fertilized trees (Fig. 3). Near the inoculation site, the concentrations of pinitol, fructose, α-glucose and β-glucose increased after N fertilization, but these differences were not significant. When all fertilization treatments were pooled, the total concentrations of soluble carbohydrates and sucrose near the inoculation site and in the outer border of the lesion were significantly lower than the corresponding values in the unwounded control (Fig. 4). In the outer border of the lesion the concentrations of fructose, α-glucose, β-glucose, sorbitol and inositol increased.

Lesion length and interrelationships with other factors studied

For this section, see also Viiri et al. (2001).

In some years, there was a modest positive correlation between lesion length and radial growth of stem or vigour in P- and NPK-fertilized trees (Table 3).

Table 4 Effect of fungal inoculation with *C. polonica* on intercorrelations among the chemical characteristics (μg mg^{-1} fresh phloem) of Norway spruce phloem and on correlation of the compounds with lesion length (mm). Spearman's rho non-parametric correlation was used. Different fertilization treatments and unfertilized control were pooled. Terpene concentrations are presented in Table 1 and stilbene concentrations in Fig. 2 in Viiri et al. (2001). *LESION* lesion length; *TERPE* total mono- and sesquiterpenes; *AGLY* total stilbene aglycones, e.g. isorhapontigenin, astringenin, resveratrol; *GLYCO* total stilbene glycosides, e.g. isorhapontin, astringin, piceid; *SUGAR* total soluble carbohydrates as in Fig. 3

		LESION	TERPE	AGLY	GLYCO
Near (n=38)[a]	TERPE	0.68**			
	AGLY	0.04	0.24		
	GLYCO	–0.38*	–0.49**	–0.16	
	SUGAR	–0.78**	–0.75**	–0.28	0.69**
Far (n=39)	TERPE	0.26			
	AGLY	0.05	0.32*		
	GLYCO	0.07	–0.43**	0.13	
	SUGAR	–0.17	–0.46**	–0.27	–0.03
Wounded	TERPE				
(n=38)[b]	AGLY		0.18		
	GLYCO		–0.13	0.31	
	SUGAR		0.01	0.33	–0.03
Unwounded	TERPE				
(n=39)[c]	AGLY		–0.06		
	GLYCO		0.18	–0.06	
	SUGAR		0.02	0.01	–0.21

*P<0.05, **P<0.01
[a] Except for GLYCO (n=33)
[b] Except for AGLY (n=35)
[c] Except for AGLY (n=34)

Near the inoculation site, the total concentration of soluble carbohydrates and the length of the lesion were negatively correlated, whereas total terpene concentration correlated positively with lesion length (Table 4). In the outer border of the lesion, lesion length did not correlate with any chemical characteristics of the phloem. Near the inoculation site, the concentration of total stilbene glycosides correlated positively with total carbohydrate concentration. In the outer border of the lesion, there was no corresponding significant correlation. On the contrary, at both isolation sites, the total concentration of terpenes showed a clear negative correlation with total concentration of soluble carbohydrates (Table 4).

Discussion

After fungal inoculation, the concentration of the major soluble carbohydrate, sucrose, decreased dramatically in the immediate vicinity of the inoculation site. The further lesion formation had progressed (wounding, fungus inoculated), the larger was the amount of total soluble carbohydrates utilized to prevent fungal invasion, which agrees with the results of Cook and Hain (1987) and Warren et al. (1999). Overall, the results clearly indicate that an active metabolizing process occurred inside the lesion. However, it is difficult to determine to what extent soluble carbohydrates were translocated to the phloem from other tissues or whether they were produced or stored at the site. In many trees, sucrose is the main translocated material in the sieve tubes of phloem. Near the inoculation sites most of the sucrose was probably processed into secondary metabolites or utilized directly by the fungus as a nutrient. When hydrolysed, sucrose yields glucose and fructose, and this reaction is not reversible. Furthermore, there are no storage reserves of fructose, glucose, sorbitol and inositol in plant tissues. Pinitol, fructose, glucose and to some extent also sorbitol and inositol might play an active role in lesion formation, because they accumulated in the outer border of the lesion. However, some carbohydrates, such as inositol and sorbitol, are used in the biosynthetic processes and cannot be direct precursors of secondary compounds in plant cells.

Together with sucrose, starch is a major reserve carbohydrate (Kozlowski and Pallardy 1997). Although the starch content of the phloem was not analysed here, it was expected to be low in the middle of August, varying in Norway spruce phloem from 4.7% (Baier 1996a) to 12.9% of dry weight in August (Horntvedt 1988). According to Horntvedt et al. (1988), the starch concentration of the phloem decreases through August to a minimum 6% of dry weight in September. According to Krekling et al. (2000), the contents of the starch granules disappear completely from August to November. In addition to seasonal variation, the amount of starch in a tree varies according to age and the location of the tissues.

Ceratocystis fungi are able to utilize many different C-based compounds as sources of nutrients. *Ceratocystis* fungi grew well on glucose and fructose, whereas the results for sucrose, sorbitol and inositol were more variable (Mathiesen-Käärik 1960). The primary fungi are able to utilize more compounds, often even di- and polysaccharides, while the secondary fungi have difficulties utilising the latter. Nevertheless, the scope of this study was not to determine whether soluble carbohydrates were utilized directly by the fungus or whether they were first converted to other soluble carbohydrates. Fungal inoculation experiments have shown that the rate of accumulation and the concentration of secondary metabolites in the lesion are crucial for successful protection. Most soluble sugars are withdrawn from or utilized around the attack-invasion site within 48 h (Cook and Hain 1987). Thus, the fungus is first confined by the consumption of essential nutrients from the entry site (Wong and Berryman 1977; Nsolomo and Woodward 1997) and only secondarily by resinosis (Raffa and Berryman 1982; Christiansen and Ericsson 1986).

In this experiment, in all fertilized trees, the radial growth of the stem responded positively to fertilization. Significant changes in the C/N ratio of needles after N and NPK fertilization were caused by an increase in N, probably not by lower availability of C. Here in the low-density inoculation experiment, the soluble carbohydrate for defence responses was not allocated at the expense of the growth of the whole stem; the responses were merely local ones. With low-density inoculation, the defence capacity of the tree is mobilized effectively and the tree

responds to fungal challenge with a discrete lesion. After mass inoculation, the resistant trees are expected to produce relatively short lesions, while susceptible trees that are overwhelmed by the fungus will produce very long necrotic lesions (Krokene and Solheim 1999).

The correlations between lesion length and the radial growth of the stem or the vigour index of N-fertilized or unfertilized trees were not significant either before or after fertilization. With other fertilization treatments, relationships were not as consistent.

The response of the radial growth of the stem to fertilization is based only on the most recent year or two. The vigour index, which reflects the history of tree growth during the past several years, is based on the amount of active sapwood that integrates the growth of the tree over at least the last 10 years. According to Waring et al. (1992), the growth efficiency of a tree requires about 2 years after treatment to respond to it. In the study of Kytö et al. (1996) when the resin ducts were counted between the wounding sites, the frequency of resin ducts was not affected by either N or P fertilization. In Norway spruce, however, traumatic resin ducts are formed in the immediate vicinity of the wounded area (Bannan 1936; Nagy et al. 2000).

Here, in to some extent nutrient-limiting conditions, no excess of soluble carbohydrates was accumulating for the production of secondary metabolites. The negative correlation between concentrations of secondary compounds and plant growth or nutrient concentrations in plant tissues have been considered to indicate a trade-off between growth and defence, as predicted in the CNB and growth/differentiation balance (GDB) hypotheses (Bryant et al. 1983; Herms and Mattson 1992). The fundamental premise of the GDB hypothesis is the existence of a physiological trade-off between growth and differentiation processes, including secondary metabolism (Herms and Mattson 1992). The GDB hypothesis subsumes the CNB hypothesis (see Herms and Mattson 1992). Furthermore, it must also be taken into account that resource-based models, such as the CNB and GDB hypotheses, can only make valid predictions concerning the total amount of C available for the production of secondary metabolites; they do not predict qualitative effects (Koricheva et al. 1998). These hypotheses cannot be used to predict plant responses in terms of individual carbohydrates, pooled CBSCs or classes of CBSCs, because resource availability does not directly affect the distribution of C at different hierarchical levels (see Fig. 2, Koricheva et al. 1998). In a broad meta-analysis of 147 studies, Koricheva et al. (1998) detected that, in terms of carbohydrates and CBSCs, plant responses to N fertilization, shading and CO_2 enrichment were consistent with predictions made with the CNB and GDB hypotheses. Soluble sugars, precursors of CBSC synthesis, were less responsive; their concentrations were only significantly affected by drought stress (increase) and shading (decrease). Nor could any clear relationship be established between the intensity of the defence reaction and the concentration of soluble carbohydrates or starch in Scots pine phloem (Croisé and Lieutier 1993) or the starch concentration in Norway spruce phloem (Christiansen and Ericsson 1986; Baier 1996b). In these previous studies, however, the concentration of starch was not measured in the immediate vicinity of the wounding site; sampling points near the wounding site varied up to 150 cm above the upper margin of the inoculation belt. More recently it has been shown that to detect relative changes in secondary compounds reliably, sampling in different growth phases and in different parts of the plant are needed (Gebauer et al. 1998; Schafellner et al. 1999).

The costs of terpenoid accumulation are high, and this obviously has negative impacts on plant fitness (Gershenzon 1994a, 1994b). In general, terpenoids have higher raw material, enzyme and storage costs than do other classes of secondary or primary plant metabolites, e.g. soluble carbohydrates (Gershenzon 1994a). Since terpenoids need complex storage structures, their synthesis may also be reduced by storage capacity rather than by the availability of C. While a high C/N ratio may increase the availability of substrate for producing defence compounds, it may not necessarily lower the other costs of chemical defence, including those of biosynthetic machinery, storage, transport and maintenance (Gershenzon 1994a, 1994b). In this experiment, however, terpenes were consistently and negatively related to the concentration of total soluble carbohydrates (this study and Viiri et al. 2001).

In conclusion, fertilization did not cause unambiguous changes in the total concentration of soluble carbohydrates in Norway spruce phloem. The concentration of total soluble carbohydrates was significantly decreased only in the outer border of the lesion in P-fertilized trees. The fertilization regimes and low inoculum density used in this study did not affect the supply of carbohydrate substrate for the production of defence compounds. Nevertheless, at the end of the experiment, the experimental trees were not suffering from a lack of any micro- or macro-nutrients, and after all fertilization treatments, the radial growth of the stem improved. The lower the amount of soluble carbohydrates present near the inoculation site the more they had been used to produce terpenes and the protective reaction lesion. In addition, near the inoculation site the strong positive correlation between the concentrations of total soluble carbohydrates and total stilbene glycosides indicates that a positive carbohydrate status favoured the synthesis of stilbenes.

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References

Baier P (1996a) Auswirkungen von Vitalität und Brutbaum-Qualität der Europäischen Fichte, *Picea abies*, und die Entwicklung der Borkenkäfer-Art *Ips typographus* (Coleoptera: Scolytidae). Entomol Gen 21:27–35

Baier P (1996b) Defence reactions of Norway spruce (*Picea abies* Karst.) to controlled attacks of *Ips typographus* (L.) (Col., Scolytidae) in relation to tree parameters. J Appl Entomol 120:587–593

Bannan MW (1936) Vertical resin ducts in the secondary wood of the Abietineae. New Phytol 35:11–46

Bryant JP, Chapin FS III, Klein DR (1983) Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. Oikos 40:357–368

Chapin FS III (1980) The mineral nutrition of wild plants. Annu Rev Ecol Syst 11:233–260

Christiansen E, Ericsson A (1986) Starch reserves in *Picea abies* in relation to defence reaction against a bark beetle transmitted blue-stain fungus, *Ceratocystis polonica*. Can J For Res 16: 78–83

Christiansen E, Waring RH, Berryman AA (1987) Resistance of conifers to bark beetle attack: searching for general relationships. For Ecol Manage 22:89–106

Cook SP, Hain FP (1987) Four parameters of the wound response of loblolly and shortleaf pines to inoculation with the blue-staining fungus associated with the southern pine beetle. Can J Bot 65:2403–2409

Croisé L, Lieutier F (1993) Effects of drought on the induced defence reaction of Scots pine to bark beetle-associated fungi. Ann Sci For 50:91–97

Franceschi VR, Krokene P, Krekling T, Christiansen E (2000) Phloem parenchyma cells are involved in local and distant defense responses to fungal inoculation or bark-beetle attack in Norway spruce (Pinaceae). Am J Bot 87:314–326

Gebauer RLE, Strain BR, Reynolds JF (1998) The effect of elevated CO_2 and N availability on tissue concentrations and whole plant pools of carbon-based secondary compounds in loblolly pine (*Pinus taeda*). Oecologia 113:29–36

Gershenzon J (1994a) Metabolic costs of terpenoid accumulation in higher plants. J Chem Ecol 20:1281–1328

Gershenzon J (1994b) The costs of plant chemical defense against herbivory: a biochemical perspective. In: Bernays EA (ed) Insect plant interactions, vol V. CRC Press, Boca Raton, Fla., pp 105–173

Herms DA, Mattson WJ (1992) The dilemma of plants: to grow or defend. Q Rev Biol 67:283–335

Horntvedt R (1988) Resistance of *Picea abies* to *Ips typographus*: tree response to monthly inoculations with *Ophiostoma polonicum*, a beetle transmitted blue-stain fungus. Scand J For Res 3:107–114

Hurlbert SH (1984) Pseudoreplication and the design of ecological field experiments. Ecol Monogr 54:187–211

Jeffers JNR (1960) Experimental design and analysis in forest research. Almqvist and Wiksell, Stockholm

Jukka L (1988) Metsänterveysopas, Metsätuhot ja niiden torjunta. Samerka Oy, Helsinki

Koricheva J, Larsson S, Haukioja E, Keinänen M (1998) Regulation of woody plant secondary metabolism by resource availability: hypothesis testing by means of meta-analysis. Oikos 83:212–226

Kozlowski TT, Pallardy SG (1997) Physiology of woody plants, 2nd edn. Academic Press, San Diego, Calif.

Krekling T, Franceschi, VR, Berryman AA, Christiansen E (2000) The structure and development of polyphenolic parenchyma cells in Norway spruce (*Picea abies*) bark. Flora 195:354–369

Krokene P, Solheim H (1999) What do low-density inoculations with fungus tell us about fungal virulence and tree resistance? In: Lieutier F, Mattson WJ, Wagner MR (eds) Physiology and genetics of tree-phytophage interactions. INRA, Paris, pp 353–362

Kytö M, Niemelä P, Annila E (1996) Vitality and bark beetle resistance of fertilized Norway spruce. For Ecol Manage 84: 149–157

Lichtenthaler HK (1999) The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. Annu Rev Plant Physiol Plant Mol Biol 50:47–65

Lorio PL Jr (1986) Growth-differentiation balance: a basis for understanding southern pine beetle-tree interactions. For Ecol Manage 14:259–273

Marcy JE, Carroll DE (1982) A rapid method for the simultaneous determination of major organic acids and sugars in grape musts. Am J Enol Vitic 33:176–177

Mason BS, Slover HT (1971) A gas chromatographic method for the determination of sugars in foods. J Agric Food Chem 19:551–554

Mathiesen-Käärik A (1960) Studies on the ecology, taxonomy and physiology of Swedish insect-associated blue stain fungi, especially the genus *Ceratocystis*. Oikos 11:1–25

Matsuki M (1996) Regulation of plant phenolic synthesis: from biochemistry to ecology and evolution. Aust J Bot 44:613–634

Mulock P, Christiansen E (1986) The threshold of successful attack by *Ips typographus* on *Picea abies*: a field experiment. For Ecol Manage 14:125–132

Münster-Swendsen M (1987) Index of vigour in Norway spruce (*Picea abies* Karst.). J Appl Ecol 24:551–561

Myers JL, Well AD (1995) Research design and statistical analysis. Erlbaum, Hillsdale, New York

Nagy NE, Franceschi VR, Solheim H, Krekling T, Christiansen E (2000) Wound-induced traumatic resin duct development in stems of Norway spruce (Pinaceae): anatomy and cytochemical traits. Am J Bot 87:302–313

Nsolomo VR, Woodward S (1997) Histological and histochemical detection of defence responses in pine embryos challenged in vitro with *Heterobasidion annosum*. Eur J For Pathol 27: 187–195

Paine TD, Raffa KF, Harrington TC (1997) Interactions among scolytid bark beetles, their associated fungi, and live host conifers. Annu Rev Entomol 42:179–206

Raffa KF, Berryman AA (1982) Accumulation of monoterpenes and associated volatiles following inoculation of grand fir with a fungus transmitted by the fir engraver, *Scolytus ventralis* (Coleoptera: Scolytidae). Can Entomol 114:797–810

Reid RW, Whitney HS, Watson JA (1967) Reactions of lodgepole pine to attack by *Dendroctonus ponderosae* Hopkins and blue stain fungi. Can J Bot 45:1115–1126

Schafellner C, Berger R, Dermutz A, Führer E, Mattanovich J (1999) Relationship between foliar chemistry and susceptibility of Norway spruce (Pinaceae) to *Phristiphora abietina* (Hymenoptera: Tenthredinidae). Can Entomol 131:373–385

Shrimpton DM (1973) Extractives associated with wound response of lodgepole pine attacked by the mountain pine beetle and associated microorganisms. Can J Bot 51:527–534

Snedecor GW, Cochran WG (1980) Statistical methods. Iowa State University Press. Ames, Iowa

Viiri H, Annila E, Kitunen V, Niemelä P (2001) Induced responses in stilbenes and terpenes in fertilized Norway spruce after inoculation with blue-stain fungus, *Ceratocystis polonica*. Trees 15:112–122

Waring RH, Thies WG, Muscato D (1980) Stem growth per unit of leaf area: a measure of tree vigor. For Sci 26:112–117

Waring RH, Savage TJ, Cromack K Jr, Rose C (1992) Thinning and nitrogen fertilization in a grand fir stand infested with western spruce budworm. Part IV. An ecosystem management perspective. For Sci 38:275–286

Warren JM, Allen HL, Booker FL (1999) Mineral nutrition, resin flow and phloem phytochemistry in loblolly pine. Tree Physiol 19:655–663

Wong BL, Berryman AA (1977) Host resistance to the fir engraver beetle. 3. Lesion development and containment of infection by resistant *Abies grandis* inoculated with *Trichosporium symbioticum*. Can J Bot 55:2358–2365

Wright E (1933) A cork-borer method for inoculating trees. Phytopathol Notes 23:487–488

Wright LC, Berryman AA, Gurusiddaiah S (1979) Host resistance to the fir engraver beetle, *Scolytus ventralis* (Coleoptera: Scolytidae). 4. Effect of defoliation on wound monoterpene and inner bark carbohydrate concentrations. Can Entomol 111:1255–1262

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