

**SURFACE STRUCTURE, WAX  
AND METHANOL-  
EXTRACTABLE COMPOUNDS  
IN SCOTS PINE AND NORWAY  
SPRUCE NEEDLES ENHANCED  
UV-B**

**HELI  
KINNUNEN**

Department of Biology

OULU 1999



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Academic Dissertation to be presented with the assent of the Faculty of Science, University of Oulu, for public discussion in Kuusamonsali (Auditorium YB 210), Linnanmaa, on 22nd June, 1999, at 12 noon.

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**Kinnunen, Heli, Surface structure, wax and methanol-extractable compounds in Scots pine and Norway spruce needles enhanced UV-B**

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***Abstract***

Increased amounts of epicuticular waxes and UV-absorbing compounds, such as flavonoids, and smaller leaf/needle surface area are plant defence mechanisms against UV-B radiation. The response of the needle epicuticular waxes of Scots pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* Karst.) seedlings to increased UV-B were investigated in short-term and long-term greenhouse experiments. In a more realistic long-term field experiment with mature Scots pines, the methanol-extractable UV-absorbing compounds were also analysed.

Some significant changes were observed in the wax tube distribution (WTD, %) and the amount of waxes in Norway spruce seedlings in the short-term Belgian greenhouse experiment (UV-B<sub>BE</sub> 0, 11.3 and 22.6 kJ m<sup>-2</sup> d<sup>-1</sup>), but no changes were detected in Scots pine seedlings. No changes in waxes were observed in the long-term Finnish greenhouse experiment (UV-B<sub>BE</sub> 0, 2.2–6.6 and 5.6–16.8 kJ m<sup>-2</sup> d<sup>-1</sup>), where both the Norway spruce and the Scots pine seedlings seemed to respond by having smaller needle surface areas. A field experiment (UV-BBE 0.5–2.4 kJ m<sup>-2</sup> d<sup>-1</sup> and 0.7–5.1 kJ m<sup>-2</sup> d<sup>-1</sup>) with mature Scots pines revealed no significant changes in WTD during the three growing seasons or the amount of waxes during the third growing season.

In the long-term field experiment the amount of UV-absorbing compounds varied significantly between seasons and/or needle age classes. Elevated amounts of these compounds were already observed in the three-day-old needles and also in the oldest (c+2) needles when the waxes were still undeveloped or already somewhat eroded. No significant differences in the amount of UV-absorbing compounds were observed between the treatments during the first and second growing seasons. During the third growing season, needles of all ages contained significantly or slightly less UV-absorbing compounds in supplemental UV-B than in the ambient treatment, possibly due to cumulative effects of UV-B in already inhibited pigment synthesis. This suggests that these defence mechanisms are not efficient enough to prevent the UV-B-induced damage in the long term.

**Keywords:** defence mechanisms, flavonoids, image analysis, scanning electron microscope



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Oulu, May 1999

Heli Kinnunen



## Abbreviations

<i>BEI:</i>	backscattered electron images
<i>c:</i>	current needles
<i>c+1, c+2...:</i>	one, two-year-old needles
<i>EDS:</i>	energy dispersive analyser
<i>UV-A:</i>	ultraviolet-A radiation (320-400 nm)
<i>UV-B:</i>	ultraviolet-B radiation (280-320 nm)
<i>UV-C:</i>	ultraviolet-C radiation (200-280 nm)
<i>SEM:</i>	scanning electron microscope
<i>WTD:</i>	wax tube distribution



## **Definitions**

*WTD (%)*: proportion of wax tubes (=wax crystallites) of the needle surface

*UV-B<sub>BE</sub>*: biologically effective UV-B radiation

*UV<sub>BE</sub>*: biologically effective UV radiation (includes UV-C radiation)



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# 1. Introduction

The amount of UV-B radiation (280–320 nm) reaching the surface of the earth is influenced by the solar zenith angle and day length, which are determined by season and latitude. Clouds, aerosols and surface albedo are also significant factors in determining the UV-B irradiation at a particular location and time (Webb 1997). The natural UV-B irradiances are highest in the summer, at low latitudes and at high altitudes (Madronich *et al.* 1995). At 68°N, for example, the biologically effective UV-B radiation varies naturally from  $0.063 \text{ kJ m}^{-2} \text{ d}^{-1}$  at midwinter (late January) up to  $4.6 \text{ kJ m}^{-2} \text{ d}^{-1}$  during the summer, whereas the maximum natural UV-B levels at 62°N and 55°N are 5.8 and  $6.9 \text{ kJ m}^{-2} \text{ d}^{-1}$  respectively (Johanson *et al.* 1995). The natural UV-B distribution has been significantly affected by the release of man-made chlorine and bromine compounds (e.g. CFCs), which result in stratospheric O<sub>3</sub> depletion and increase the amount of UV-B radiation reaching the earth's surface (Madronich *et al.* 1995). Relative to 1960, the maximum ozone depletion expected at the northern mid-latitudes is 12–13% in winter/spring and 6–7% in summer/fall, while at the southern mid-latitudes it is about 11% in all seasons (Madronich *et al.* 1995).

Plants have developed different defence mechanisms against UV-B radiation, such as thicker and smaller leaves (Sullivan & Teramura 1988, Bornman & Vogelmann 1991), increased production of UV-absorbing compounds (such as flavonoids) (Caldwell *et al.* 1983, Tevini *et al.* 1991) and higher amounts of reflective waxes (Steinmüller & Tevini 1985, Dubé & Bornman 1992, Barnes & Cardoso-Vilhena 1996, see also Rozema *et al.* 1997a). The protective capacity of epicuticular waxes and epidermal screening pigments is based on their ability to moderate the wavelengths of light that penetrate into the tissues (Robberecht *et al.* 1980, Vogelmann 1993). Flavonoids are phenolic compounds which are synthesized via the phenylpropanoid pathway (Hahlbrock & Grisebach 1979, Koes *et al.* 1994). Flavonoids are situated in the epidermis and strongly absorb UV-B radiation transmitting visible or photosynthetically active radiation (Hahlbrock & Grisebach 1979, Caldwell *et al.* 1983, Robberecht & Caldwell 1986, Day *et al.* 1992, 1993, Jordan 1996, Krauss *et al.* 1997, Allen *et al.* 1998, Jansen *et al.* 1998). Waxes are a complex mixture of hydrophobic lipids that do not significantly absorb UV-B radiation (Jordan 1996, Rozema *et al.* 1997b), but increased wax on leaf surface may reduce UV-B penetration due increased reflection from the leaf surface, although reflection for most leaves is usually

not more than 10% (Robberecht *et al.* 1980). In some species, various phenolics can be contained in the cuticle, and these may be important UV-B absorbers (Rozema *et al.* 1997b). There are a few species which do exhibit leaf surface reflectance of the order of 20 to 70% in the UV waveband. This has been reported for some species that have a dense glaucous surface, such as *Picea pungens* (Clark & Lister 1975) or a dense pubescence surface (Robberecht *et al.* 1980). Hairs and trichomes may also increase reflection (Karabourniotis *et al.* 1992, 1995, Karabourniotis & Fasseas 1996, Baldini *et al.* 1997). According to Barnes and Cardoso-Vilhena (1996), waxes and cuticular membrane may be of direct significance in attenuating UV, but some authors suggest that the role of the epicuticular wax layer itself is not so important, because the removal of epicuticular waxes did not significantly reduce screening (Bornman & Vogelmann 1988, Day *et al.* 1992). In cold climates, no new epicuticular waxes are produced after the first growing season (Hallam 1982, Percy *et al.* 1993), and the increased production of UV-absorbing compounds might therefore be more effective as a long-term defence mechanism. However, waxes and leaf hairs are important in the natural adaptation to UV-B radiation (Clark & Lister 1975, Karabourniotis *et al.* 1993), and they may protect potentially sensitive surface targets, such as guard cells or cells lining the substomatal cavity (Day *et al.* 1992).

The differences in UV-B sensitivity between plant species may be partly explained by differences in the capacity of screening UV-B radiation, which depends on the concentration, spatial location and quality of UV-B-absorbing compounds as well as the leaf anatomy (Day 1993, Vogelmann 1993, Bornman *et al.* 1994, Karabourniotis & Fasseas 1996, Sullivan *et al.* 1996, McLeod & Newsham 1997). For example, blue light (460 nm) and UV-A (360 nm) penetrated much further into spruce (*Picea engelmannii*) than fir (*Abies lasiocarpa*) needles, probably due to differences on pigmentation and leaf anatomy (Bornman & Vogelmann 1988). Species grown in naturally high UV-B radiation environments are acclimated to these growing conditions by better developed defence mechanisms (Robberecht *et al.* 1980, Kossuth & Biggs 1981, Caldwell *et al.* 1982, Beggs *et al.* 1986, Sullivan *et al.* 1992, Wand 1995, Krauss *et al.* 1997). Conifer needles are more tolerant of UV-B than deciduous leaves due to their thick waxy cuticle, small needle surface area and higher amount of UV-absorbing pigments (Sullivan & Teramura 1988, Day 1993). For example, UV-B transmission was 0.5–1% in loblolly pine (*Pinus taeda*) but 10–20% in sweetgum (*Liquidambar styraciflua*) (Sullivan *et al.* 1996). The better screening ability of conifers may be an evolutionary response to potentially large life-time doses of UV-B (Day *et al.* 1992, Vogelmann 1993). However, although fully developed conifer needles can attenuate almost all of the incident UV-B radiation, the screening ability of developing conifer needles is not equally effective, making the young needles more sensitive to UV-B (Day *et al.* 1992, DeLucia *et al.* 1992, Naidu *et al.* 1993).

The defence mechanisms do not provide complete protection against increased UV-B, because their effectiveness depends on environmental factors and the developmental stage of the plant (Caldwell *et al.* 1983, Wand 1995). In case these defence mechanisms do not work effectively, UV-B may cause damages in the DNA, photosynthesis, membrane functioning, morphology, phenology and chemical composition of plants (see, for example, Krupa & Kickert 1989, Runeckles & Krupa 1994, Rozema *et al.* 1997a). To some extent, plants are able to repair DNA damage by the phytolyase enzyme system (Taylor *et al.* 1997). The repair mechanisms and flavonoid synthesis work better in natural light conditions, and plants in field conditions are therefore more resistant to UV-B (Beggs *et al.*

1986, Caldwell *et al.* 1994, 1995). Up till now, most of the UV-B experiments have been greenhouse or chamber experiments, where the responses have been overestimated partly due to unnaturally low visible light intensity and the distorted balance between UV-B, UV-A and PAR (Mirecki & Teramura 1984, Cen & Bornman 1990, Fernbach & Mohr 1992, Caldwell *et al.* 1994, Kim *et al.* 1996). Greenhouse-grown plants have thinner and less well developed cuticles than field-grown plants (Martin & Juniper 1970) and might thus show greater sensitivity (Runeckles & Krupa 1994). Up till now, only six conifer species (*Pinus halepensis*, *Pinus pinea*, *Pinus taeda*, *Pinus contorta*, *Picea abies* and *Picea engelmannii*) have been studied in field sites, and only *Pinus taeda* has shown UV-B-induced growth reductions (see Laakso & Huttunen 1998, Fischbach *et al.* 1999). The earlier field experiments have been done on conifer seedlings, which may react differently compared to mature trees (Björn 1996). As far as we know, there are no earlier field experiments done with mature Scots pines. A survey of some 300 papers from the past 20 years indicated that about 80% of the UV-B experiments have been short-term experiments (a few months or less) (Rozema *et al.* 1997b), but in long-lived and slow-growing conifers several years of exposure are probably needed to yield reliable information about their ability to acclimate to increased UV-B.

The special features of the northern climate may be problematic to the plants and their ability to tolerate increased UV-B radiation. Although the UV doses are smaller at the northernmost latitudes than at lower latitudes, the nearly 24-h photoperiod in the summer results in total daily radiation values almost identical to those at other locations (Caldwell *et al.* 1980). Possibly even 30% higher UV-B doses during the spring months (Jokela *et al.* 1995) may be harmful to the northern plants because the reflectance from snow may significantly increase the UV-B dose received by trees (DeLucia *et al.* 1991), which may be crucial for postdormant trees at the beginning of gas exchange (Laakso & Huttunen 1998). At low temperatures, the chemical defence mechanisms may work ineffectively, as has been documented with the repair mechanisms (Björn *et al.* 1997).



## 2. Aim of the research

The aim of this work was to gain an understanding of the responses of Norway spruce and Scots pine to enhanced UV-B radiation and their defence mechanisms against UV-B (I-VI). For this purpose, short-term and long-term greenhouse pilot experiments were carried to obtain preliminary information about effects of increased UV-B on our conifer seedlings (I, II) before the long-term field experiment was established (III-VI). In both greenhouse experiments, UV-induced changes in the needle surface area, amount of waxes and wax tube distribution were studied (I, II), and in the field experiment, the methanol-extractable UV-absorbing compounds were also analysed (III, V, VI).

Studies were carried out

1. to describe the UV-B-induced changes in Scots pine needle waxes with a new phase-analysing tool. (I)
2. to identify the differences in the responses of wax structure and wax amount to increased UV-B in short- and long-term greenhouse experiments with seedlings of spruces and pines from several provenances. (II)
3. to present the modified image-analysis system and its ability to score the epicuticular waxes of developing and full-developed needles of mature Scots pines exposed to supplemental UV-B. (V)
4. to identify the possible cumulative changes in the development of wax structure and and the accumulation of UV-absorbing compounds in the different needle age classes during 1–3 growing seasons and to identify the variation in responses between the different years. (III–VI)



### **3. Materials and methods**

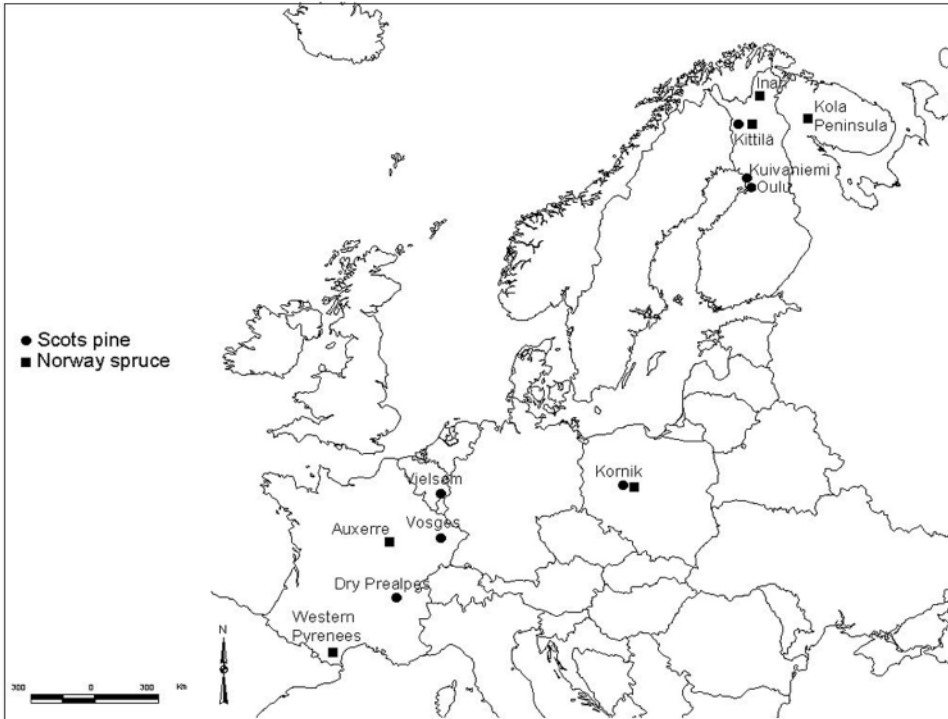
#### **3.1. Short-term greenhouse experiment**

The plant material in the short-term Belgian greenhouse experiment consisted of four provenances of 3- to 4-year-old nursery-grown Scots pine (*Pinus sylvestris* L.) from latitudes 45°N to 65°N and three provenances of nursery-grown seedlings and one of cloned Norway spruce (*Picea abies* Karst. L.) from latitudes 45°N to 52°N (Fig. 1). The seedlings were planted in a standardized soil mixture consisting of sand, unfertilized peat and composted soil. The seedlings were randomly distributed into the treatment categories, with three or four trees per provenance per dose treatment. The experiment was conducted during 13 weeks from April 13 till July 13, 1994, in the Faculty of Agricultural Sciences of Gembloux, Belgium. The temperature in the greenhouses was regulated between +16°C and +20°C in ambient light conditions. To simulate UV enhancement conditions, the plants were irradiated with UV-lamps (Philips UV-B TL 12 40 W), using four lamps in each experimental area. The UV-C radiation was filtered out by cellulose diacetate filters (Courtaulds Ltd, 95 mm). The biologically effective doses (UV-BBE) were 0, 11.3 and 22.6 kJ m<sup>-2</sup> d<sup>-1</sup>. The UV measurements were done with a Jobin & Yvon UV spectroradiometer from the Institute for Spatial Aeronomy (Brussels, Belgium) (II).

*Table 1: Summary of the three enhanced UV experiments.*

Experiment	UV doses (kJ m <sup>-2</sup> d <sup>-1</sup> )	Plant material	Sampling	Variables	Method	Study
Greenhouse, UV-B: Belgium, 13 weeks	0 11.3 22.6	Scots pine and Norway spruce seedlings	end of the experiment	WTD and amount of waxes	SEM, ocular colorimetric method	II II
Greenhouse, UV-B+ Finland, UV-C: two-growing-season	0 02.2–6.6 5.6–16.8	Scots pine and Norway spruce seedlings	end of the experiment	WTD and amount of waxes	image analysis colorimetric method	I,II II
Field experiment Oulu (65°N), three- growing-sea- son	0.5–2.4 0.7–5.1	Mature Scots pines	1996: twice in a growing season end of the growing season	WTD pigments	image analysis methanol extraction	III IV
			1997: 3 times in a week (15 times) 3 times during the growing season	WTD pigments	image analysis methanol extraction	III V
			1998: twice in a month (6 times) twice in a month (6 times) twice in a month (6 times)	WTD pigments amount of waxes	image analysis methanol extraction gravimetric method	VI VI VI

**Fig. 1. Provenances of the studied Scots pine and Norway spruce seedlings (I, II).**



### 3.2. Long-term greenhouse experiment

Two provenances of 2- to 4-year-old nursery-grown Scots pine and Norway spruce seedlings and a grafted clone of Scots pine were used. All the seedlings were from northern latitudes (65°N–69°N) (Fig. 1). The seedlings were planted in pots containing forest soil and sand (7:1). The plants were randomly distributed into the treatment categories, with six trees per provenance per dose treatment. Supplementary UV radiation was given in a greenhouse for seven days a week from 11 June to 30 September 1994 and from 1 February to 30 September 1995, using Philips Ultraviolet TL 100 W/01 lamps. The biologically effective UV-doses (UV-B,  $C_{BE}$ ) were 0, 2.2–6.6 and 5.6–16.8  $\text{kJ m}^{-2} \text{d}^{-1}$  (II).

### 3.3. Field experiment

The field site for the study was located near the University of Oulu (65°N) (Fig. 1). A lamp bank consisting of three lamps (Philips TL 12/40 W) was mounted 50 cm above a selected branch of each Scots pine. The pines were about 40 years old, and were allocated to ambient (7 trees) and supplemental UV-B (7 trees) treatments. The lamps were filtered either with 0.1 mm cellulose-diacetate film (transmission down to 290 nm) for

supplemental UV-B or with 0.1 mm polyester film (absorbs almost all radiation below 316 nm) for ambient treatment. The filters were changed once in a month. The lamps were on for 6 hours per day centered at solar noon on 7 days a week. The UV-B doses were adjusted to follow the normal UV-B doses, which vary during the day and the growing season (Jokela *et al.* 1995). The daily UV-B doses were regulated by a potentiometer to give different UV-B fluence rates without causing spectral displacement. Spectral irradiances under the lamps were measured at least once a week with a spectroradiometer (Model 754, Optronics, Orlando, FL, USA) at 1 nm intervals. The absolute spectral UV irradiance of the Philips lamp was weighted with the generalized plant response action spectrum (Caldwell 1971) and normalized to 300 nm to obtain the daily biologically effective irradiance (UV-B<sub>BE</sub>). UV-B doses varied in the ranges of 0.5–2.4 kJ m<sup>-2</sup> d<sup>-1</sup> (ambient) and 0.7–5.1 kJ m<sup>-2</sup> d<sup>-1</sup> (supplemental UV-B) (Table 2). Solar radiation (305–1500 nm) was measured with a pyranometer CM-3 (Kipp & Zonen). In the first year, 1996, the exposure was started on June (III) and in the other two years at the beginning of April (IV, V, VI). In each year, exposure was discontinued at the end of September.

Table 2: The monthly means of UV-A and biologically effective UV-B doses in the field experiment during the three growing seasons. Solar radiation and temperatures are expressed as monthly means and maximum values. .

	UV-A (W/m <sup>2</sup> )		UV-B <sub>BE</sub> (kJ m <sup>-2</sup> d <sup>-1</sup> )		Solar radiation (W/m <sup>2</sup> )		Temp (°C)		Prepic (mm)
	amb.	UV-B	amb.	UV-B	mean	max	mean	max	
1996:									
June	30.6	32.6	2.4	5.1	206	808	13.1	19.4	22
July	23.9	26.8	1.1	3.7	186	864	15.4	23.0	48
August	22.0	24.8	1.1	2.4	190	627	16.4	24.3	19
September	11.8	13.1	0.5	0.7	90	480	7.5	13.6	8
1997:									
April	*	*	*	*	171	652	-2.4	2.4	40
May	24.4	24.8	1.8	4.4	191	820	5.3	10.1	20
June	28.4	29.5	1.9	4.2	255	782	4.5	20.5	33
July	24.4	25.1	1.4	3.6	228	795	18.7	24.6	55
August	21.1	22.5	1.3	2.1	182	648	15.4	20.6	43
September	14.2	14.1	0.7	0.9	80	490	9.4	13.5	73
1998:									
April	*	*	*	*	62	614	-1.1	4.3	10
May	24.0	27.4	1.2	4.0	170	697	6.1	11.4	41
June	26.8	27.4	1.8	4.7	197	786	13.3	18.4	67
July	26.8	25.0	1.3	4.0	165	850	17.0	22.5	90
August	18.9	18.8	0.8	1.7	111	612	12.9	17.0	81

\* = data not available

### 3.4. Wax response

For the SEM investigation, the needles were collected with tweezers and they were packed loosely to avoid compression. The samples were stored in a freezer until analysed. The middle parts of the needles were mounted on brass stubs and sputtered with gold-palladium on the next day (Polaron E5100).

In the Belgian experiment, the current-year needles (about 3 months old) were collected at the end of the experiment and micrographed (magnification 200x) (3/seedling) under a scanning electron microscope (JEOL JSM 6400) with a 12 kV power source. The wax structures of pine needles were scored using a classification method described by Turunen and Huttunen (1991). The epistomatal tubular waxes of spruce needles were classified by the method of Sauter *et al.* (1987). In the long-term Finnish greenhouse experiment, the current-year needles (about 5 months old) were collected at the end of the experiment and the wax tube distribution (WTD, %) of the pine and spruce needles (3

needles/seedling) was studied under a SEM with the help of a phase-analysing program of an EDS (LINK eXL) analyser. To increase the contrast between the areas covered by wax tubes and the eroded areas, processed (edge sharpen, erode and dilate) backscattered electron images (BEIs) were used. The WTD was scored as a percentage of the needle surface area from BEI micrographs of magnification 1300x (Manninen *et al.* 1996a,b, I).

Current-year needle samples (5/tree) from the long-term field experiment were taken twice in a growing season in 1996. In the other two growing seasons, needle samples (5/tree) were collected from the beginning of needle development to fully-grown needles three times/week in 1997 (15 samplings) and twice a month in 1998 (6 samplings). At the end of the growing season, all the previous-year needles which had developed during the experiment (c+1 and c+2 needles) were collected. The samples were analysed using the phase analysis method (Manninen *et al.* 1996a,b, I) or a slightly modified image analysis method (V, VI). The needle samples collected in 1997 were analysed with this new image analysis method and also with the wax classification method (Turunen & Huttunen 1991) (V).

In both greenhouse experiments, the quantity of chloroform-soluble waxes on Scots pine and Norway spruce needles (4–6 needles/seedling) was determined by the colorimetric method (Ebercon *et al.* 1977) with the exception that carbowax 3000 was used as a standard. The absorbances were measured at 590 nm by a spectrophotometer (Beckman DU, -64). Needle dry weight and length were measured to express the results per needle dry weight (mg/g) and surface area (mg/cm<sup>2</sup>). Needle surface area was calculated according to Flower-Ellis and Olsson (1993) for Scots pine and according to Riederer *et al.* (1988) for Norway spruce. Needle waxes (5 needles/tree) from the long-term field experiment were analysed only in 1998 using the modified method of Schuck (1976). Current-year needles were sampled 6 times and in the last sampling previous-year needles (c+1 and c+2 needles) were also sampled. The wax amount was expressed as per needle surface area (mg/cm<sup>2</sup>).

### 3.5. Methanol-extractable pigments

The current-year needle samples for pigment analysis were taken only from the field experiment, once in 1996, three times in 1997 and twice a month in 1998 (6 samplings). In the last sampling in 1998 also the c+1 and c+2 needles were sampled. The needle samples were stored at -70°C until analysed. From each needle pair, one needle was used for pigment analysis and the other for needle length measurements, to allow the concentrations to be expressed on a needle area basis (Flower-Ellis & Olsson 1993). The needles were cut into smaller pieces and extracted in 15 ml of methanol, HCl and distilled H<sub>2</sub>O (90:1:1, v/v/v). The solutions were stirred and heated (60°C) for 10 min, cooled at room temperature for 15 min and filtered through 90 mm screens (Day 1993). The concentrations of UV-absorbing compounds were estimated by measuring absorbances at 300 nm (in 1996) or at 320, 310, 300, 290 and 280 nm (in 1997 and 1998) using a UV/visible spectrophotometer (UV-160 A, Shimadzu).

### **3.6. Statistics**

The statistical analyses were performed using SPSS (SPSS Inc., Ver 6.1), to test the significance of the differences between the treatments, the provenances and the needle ages. The differences were tested using one-way analysis of variance (ANOVA) or a non-parametric test (Kruskall-Wallis). Two-way ANOVA was used to test the interactions between the needle ages/provenances and the treatments. Differences in the mean values were regarded as significant at the levels of  $P < 0.05^*$  and  $P < 0.01^{**}$ .



## 4. Results

### 4.1. Seedlings

#### *4.1.1. Norway spruce*

Enhanced UV-B had no effect on the needle surface area in the short-term Belgian experiment, whereas in the long-term Finnish experiment the Kittilä spruces had 35% smaller needle surface area (ANOVA,  $P = 0.003$ ) in the  $2.2\text{--}6.6 \text{ kJ m}^{-2} \text{ d}^{-1}$  UV treatment compared to the control needles, and the same trend was also observed in the Kuivaniemi spruces.

The amount of chloroform-soluble waxes on spruce needles varied within  $30.3\text{--}102.0 \text{ mg/g}$  needle dry weight (short-term greenhouse experiment) and  $32.1\text{--}42.2 \text{ mg/g}$  needle dry weight (long-term greenhouse experiment). In the short-term experiment, the responses varied clearly within the provenances. While the spruces from the Dry Préalpes (France, mountain) provenance had an increased amount of waxes in the  $22.6 \text{ kJ m}^{-2} \text{ d}^{-1}$  treatment (ANOVA,  $P = 0.241$ ), the spruces from Kornik (Poland) ( $P = 0.008$ ) and Vosges (France, plain) ( $P = 0.087$ ) had a decreased amount of waxes when exposed to the same UV dose (II).

In both greenhouse experiments, the wax structure of spruce needles was well developed and the needles showed only slight structural degradation of the surface wax (see Fig. 4 in paper II). The wax tube distribution (WTD, %) of spruce needles varied within  $92\text{--}100\%$  (short-term greenhouse experiment) and  $64\text{--}77\%$  (long-term greenhouse experiment). The only significant UV-B-induced change was observed in the short-term experiment on needles from the Kornik (Poland) provenance, which had significantly (ANOVA,  $P = 0.046$ ) decreased WTD at the highest UV dose ( $22.6 \text{ kJ m}^{-2} \text{ d}^{-1}$ ).

### 4.1.2. Scots pine

The needle surface area of Scots pines varied clearly within the provenances in the Belgian short-term experiment, but no trend was observed between the treatments. In the long-term Finnish experiment, the highest UV dose ( $5.6\text{--}16.8 \text{ kJ m}^{-2} \text{ d}^{-1}$ ) caused a 20% smaller surface area (ANOVA,  $P = 0.013$ ) in the Kola Peninsula provenance, and slight reductions were also observed in the Kittilä provenance.

The amount of waxes (mg/g needle dry weight) varied within 18.1–36.6 (short-term experiment) and 25.8–40.8 (long-term experiment) (see Fig. 2 in paper II). Enhanced UV-B had no significant effect on the amount of waxes of pine needles in neither of the two greenhouse experiments.

SEM micrographs revealed that the needle wax synthesis in the short-term Belgian experiment was poor or prevented, especially in the northernmost, Finnish provenance (II), although the WTD values between these two experiments varied only slightly (due to the different analysing methods). The WTD of pine needles varied within 32–62% (short-term experiment) (II) and 28–66% (long-term experiment) (I, II), and no significant changes between the treatments were observed.

## 4.2. Mature Scots pines

The needle surface area of mature Scots pines was not influenced by supplemental UV-B during the three growing seasons of exposure.

In the field experiment, the amount of waxes was determined with the gravimetric method, and it varied in the range  $0.1\text{--}0.8 \text{ mg/cm}^2$  (or  $15.5\text{--}98.6 \text{ mg/g}$  needle dry weight) (VI). Supplemental UV-B had no significant effect on the amount of waxes, but the amount changed between the needle age classes. In the youngest, three-day-old needles, no chloroform-soluble waxes were yet detected (possibly due to the small sample size), whereas the maximum amount of waxes was reached after only two weeks. The least waxes were observed in the oldest (c+2) needles (VI).

Supplemental UV-B caused no changes in WTD (%) during the three growing seasons (III, V, VI). In different-aged needles, WTD varied significantly due to the natural development and erosion of the wax structure. No tubular waxes were detected in three-day-old needles, but after two weeks already, WTD was 50% (ambient) and 53% (supplemental UV-B) of the needle surface covered by wax tubes (VI). Slight differences were observed in the WTD values between the three growing seasons, due to the different weather conditions (Table 2), although the differences in environmental conditions seemed to have no significance on the responses to supplemental UV-B (Fig. 2). In 1997, the WTD of one-week-old needles was 26–29% (V), whereas in 1998 the WTD of three-day-old needles was as high as 50–53% (VI). In late September, WTD was 56–61% in 1997 and 57–62% in 1998, but only 48% in 1996. Supplemental UV-B caused no changes in the developmental stage of the needles or the erosion rate of waxes (Fig. 2).

The amount of UV-absorbing compounds did not significantly differ between the ambient and supplemental UV-B treatments during the first and second growing seasons UV-B exposure (IV). In the second growing season, the younger needles (one- and two-

month-old) contained less of these compounds in supplemental UV-B, whereas the older needles (11- and 14-month-old) had slightly more UV-absorbing compounds in supplemental UV-B compared to the ambient treatment (IV). During the third growing season, the needles exposed to supplemental UV-B had significantly (ANOVA,  $P=0.033$  and  $0.024$ ) lower amounts of UV-absorbing compounds ( $A_{310,320}/\text{cm}^2$ ) than the ambient one-month-old needles (15 July) (VI). In the other needle age classes and at the other wavelengths ( $A_{280-300}/\text{cm}^2$ ), too, needles had slightly more UV-absorbing compounds in the ambient treatment (Fig. 3).

During the second growing season, there was a slight trend towards an increase of UV-absorbing compounds along with increasing needle age in the first part of the UV-B range (280 nm), whereas no clear trend was observed in the middle of the UV-B range (300 nm) (IV). During the third growing season, the amount of these compounds varied between the needle development stages (VI). In three-day-old needles (18 June), the amount of UV-absorbing compounds was high, but it later decreased rapidly. The lowest level was reached in two-month-old needles (in August), whereas in September (c, c+1 and c+2 needles) the amount of these compounds was elevated again and the highest amount was observed in the oldest (c+2) needles (VI).

In the different growing seasons, the amounts of UV-absorbing compounds were similar in the one- and three-month-old needles collected in July and September. In August 1997 the amount of UV-absorbing compounds ( $A_{300}/\text{cm}^2$ ) was 0.099 (ambient) and 0.114 (supplemental UV-B), but in August 1998 only 0.048 and 0.047, respectively, although in 1998 the August samples were taken two weeks earlier than in 1997. The summer 1997 was warm and sunny, while the summer 1998 was cool and rainy (Table 2).

Fig. 2. The wax tube distribution (WTD, %) of the needles of mature Scots pines under ambient and supplemental UV-B during the exposure years 1996–1998. The error bars represent SD (n=7).

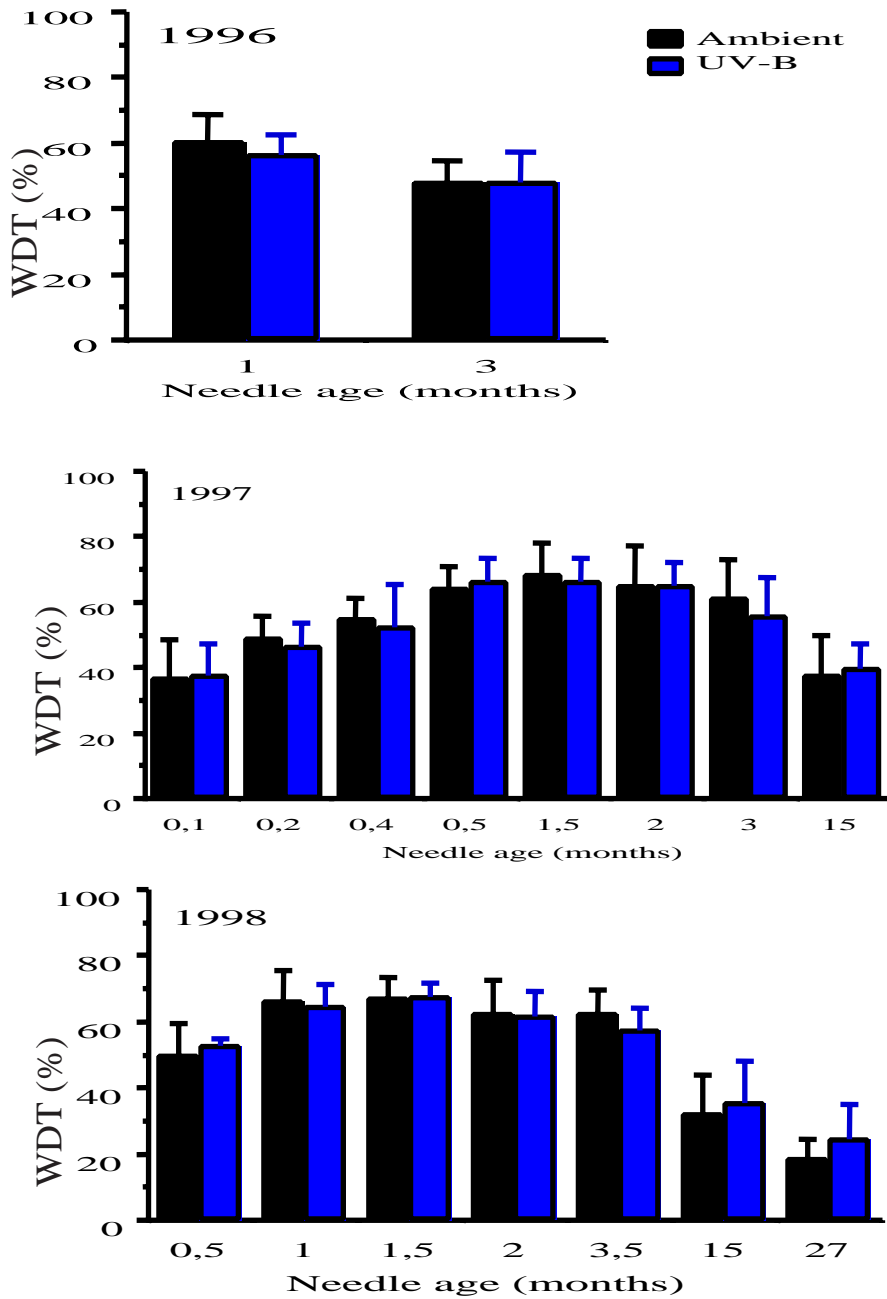
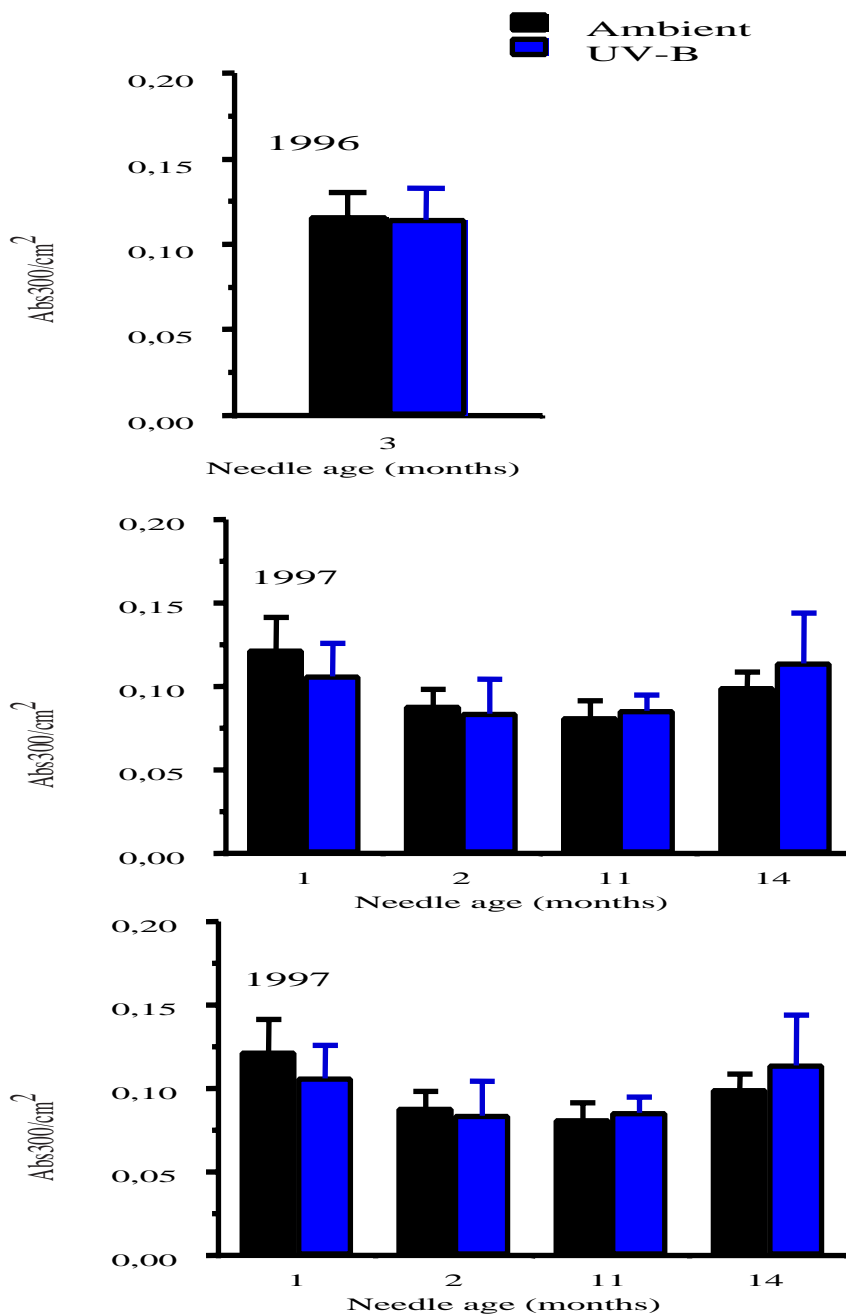


Fig. 3. Methanol-extractable UV-absorbing compounds ( $A_{300}/\text{cm}^2$ ) in the needles of mature Scots pines under ambient and supplemental UV-B during the exposure years 1996–1998. The error bars represent SD ( $n=7$ ).





## 5. Discussion

### 5.1. Wax response

#### *5.1.1. Seedlings in greenhouse experiments*

Increased UV-B caused significant changes in the wax amount and WTD of spruce needles in the short-term Belgian greenhouse experiment. The responses varied between the provenances, so that the spruces from Dry Préalpes (France, mountain) were able to increase their wax production even under the highest UV-B dose (UV-B<sub>BE</sub> 22.6 kJ m<sup>-2</sup> d<sup>-1</sup>), whereas the spruces from Kornik (Poland) and Vosges (France, plain) had clearly decreased wax amounts under the same dose (II). These mountain spruces seemed to be better acclimated to higher UV-B doses (Larson *et al.* 1990, Sullivan *et al.* 1992, Ziska *et al.* 1992). Because the lower UV-B dose (UV-B<sub>BE</sub> 11.3 kJ m<sup>-2</sup> d<sup>-1</sup>) caused no marked changes in wax production, it might be concluded that the critical level for wax synthesis of the spruces acclimated to lower UV-B doses was 11.3–22.6 kJ m<sup>-2</sup> d<sup>-1</sup>. The critical level for the wax biosynthesis of sugar maple (*Acer saccharum*) has been suggested to be lower (UV-B<sub>BE</sub> 6.2 kJ m<sup>-2</sup> d<sup>-1</sup>) (Gordon *et al.* 1998b), which is understandable in view of the fact that deciduous species are considered to be more sensitive to UV-B than conifers (Sullivan & Teramura 1988, Day 1993). The lower UV doses used in the long-term Finnish greenhouse experiment (UV<sub>BE</sub> 2.2–6.6 kJ m<sup>-2</sup> d<sup>-1</sup> or 5.6–16.8 kJ m<sup>-2</sup> d<sup>-1</sup>) (II) revealed no changes in the wax amount or WTD. These results could be taken to indicate that needles may increase wax production as a short-term response to increased UV-B, but other defence mechanisms are more useful as a long-term response. In this long-term Finnish experiment, for example, seedlings protected themselves by having smaller needle surface area (II) and/or thicker epidermis (Laakso *et al.* 1996). It is not always obvious whether the unaffected wax production indicates an inability to produce more waxes in those conditions or whether there is no need for increased wax production. At least in this long-term greenhouse experiment, the latter alternative is more obvious, since enhanced UV was even beneficial for Scots pines, causing increased height growth and radial growth (Laakso 1997). The development of waxes is determined genetically and

modified by many environmental factors, such as light quantity and quality, day length, temperature and relative humidity (Jeffree 1986, Cape *et al.* 1989, Cape & Percy 1993) which generally differ in greenhouses from the outdoor conditions, and the responses cannot therefore be directly extrapolated to nature.

### ***5.1.2. Mature trees in field conditions***

The more realistic field experiment (UV-B<sub>BE</sub> 0.7–5.1 kJ m<sup>-2</sup> d<sup>-1</sup>) with mature Scots pines revealed no UV-B-induced changes in the wax structure (III, V, VI) during the three growing seasons of exposure or in the wax amount (VI) during the third growing season. The xeromorphic waxy needles in the northern climate are possibly well protected against unfavorable conditions, such as high radiation, drought, heat, wind, snow, frost, pathogens and air pollutants (Martin & Juniper 1970, Hanover & Reicosky 1971, Günthard-Goerg 1986) without additional wax production. Furthermore, the mature pines had a more xeromorphic needle structure with thicker cell walls than the seedlings (Walles *et al.* 1973, Esau 1977, Huttunen *et al.* 1989), indicating the better ability of mature trees to tolerate increased UV-B radiation. Needles from mature Scots pines contained higher amounts of waxes compared to pine seedlings (II, VI). Hence, more abundant production of waxes might be unfeasible, whereas changes in the wax chemical composition or other defence mechanisms might be more beneficial.

### ***5.1.3. Comparison between the greenhouse and field experiments***

It is already well known that the responses to UV-B in chamber and greenhouse experiments are often overestimated, whereas only slight UV-B-induced changes in field conditions occur generally. The greater sensitivity to UV-B in greenhouse and chamber experiments can be partly explained by the much thinner and less developed cuticle compared to field-grown plants (Runeckles & Krupa 1994). In the present study, too, the needle wax structure of field-grown Scots pines was better developed and they had higher WTD (III, V, VI) than the greenhouse-grown pines (II), although part of the differences might be due to the different-aged research material (seedlings vs. mature trees). In chambers and greenhouses, the light conditions are unnatural and therefore the synthesis of UV-B-absorbing compounds and the repair mechanisms might also be insufficient (Beggs *et al.* 1986, Caldwell *et al.* 1994, 1995). The poorly developed wax structure together with the insufficient pigment synthesis (not analysed) could partly explain the UV-B-induced changes in needle length and seedling growth in the greenhouse experiments, whereas no such distinct changes were observed in field conditions.

### 5.1.4. Different analysing methods

Scanning electron microscopic classification methods (e.g. Cape & Fowler 1981, Huttunen & Laine 1983, Crossley & Fowler 1986, Tuomisto 1988, Turunen & Huttunen 1991, 1996, Hellqvist *et al.* 1992, Tuomisto & Neuvonen 1993, Ylimartimo *et al.* 1994, Trimbacher & Eckmüller 1997, see also V) mainly focus on the visual appearance and degradation of epicuticular waxes, whereas UV-B has been suggested to cause increased production of waxes (Barnes & Cardoso-Vilhena 1996). In our preliminary chamber experiment, the classification method (Turunen & Huttunen 1991) revealed a significant increase in wax tubes in the needles of UV-treated Scots pine seedlings (Kinnunen *et al.* 1994). Although the responses can be partly explained by the unnaturally low level of visible light in the chamber (Mirecki & Teramura 1984, Cen & Bornman 1990), the results proved that an increased amount of wax tubes caused by enhanced UV-B is detectable by a scanning electron microscope. The classification methods are subjective and inexact, and therefore a new phase analysing method was developed. By this method, the wax tube distribution (WTD) is scored as a percentage of the needle surface area at a certain (1300x) magnification (I). Later, a slightly modified image analysis method was compared to the classification method (V). This image analysis proved to be a quick, easy, less subjective and more exact method, and the numerical values given by the analysis make the data more comparable and easy to analyse statistically compared to the classification methods. The image analysis method is valid for all conifers and also for scoring the effects of air pollutants.

The epicuticular wax structure as a response to increased UV-B has been studied earlier in cucumber (*Cucumis sativus*) (Tevini *et al.* 1983), bean (*Phaseolus vulgaris*), barley (*Hordeum vulgare*) (Steinmüller & Tevini 1985), tobacco (*Nicotiana tabacum*) (Barnes *et al.* 1996), beech (*Fagus sylvatica*), holm oak (*Quercus ilex*) (Paoletti 1998) and sugar maple (*Acer saccharum*) (Gordon *et al.* 1998b). Clark and Lister (1975) studied the wax structure of Douglas fir (*Pseudotsuga menziesii*), Sitka spruce (*Picea sitchensis*), Colorado spruce (*Picea pungens* Engelm.) and Blue spruce (*Picea pungens* Engelm. var. *hoop-sii*) needles to explain the natural adaptation to different UV doses, but this study did not involve any experimental data. As far as we know, there are no earlier UV-B experiments on UV-B-induced changes in the wax structure of conifer needles, but the amount of needle waxes or wax chemistry have been studied in two UV-B experiments. In *Pinus pinea*, an increasing trend (ca 11%) in epicuticular waxes (% of dry weight) was observed under supplemental UV-B (maximum UV-B<sub>BE</sub> 11.21 kJ m<sup>-2</sup> d<sup>-1</sup>) (Manetas *et al.* 1997). A short-term UV-B exposure (UV-B<sub>BE</sub> 0.61–5.99 kJ m<sup>-2</sup> d<sup>-1</sup>) in controlled environment cabinets revealed no changes in the epicuticular wax production (mg g<sup>-1</sup>) in four *Picea* species, whereas the wax composition was altered in *Picea abies*, *P. rubens*, *P. mariana* (Gordon *et al.* 1998a). In this study, increased UV-B caused a significantly increased relative quantity of nonacosane diols, which together with other secondary alcohols, comprised the tube crystallites (Holloway 1994, Percy *et al.* 1994), which are more reflective than the amorphous waxes (Percy *et al.* 1992). Hence, the essential factor in determining the waxes' ability to act as a UV-B filter does not seem to be the total amount of waxes, but rather the wax structure, which is largely determined by their chemical composition (Baker 1982, Garrec 1994, Percy *et al.* 1994).

The mechanism by which UV-B alters the wax chemical composition is unknown, but there is some evidence that UV-B radiation influences specific enzymes involved in the wax biosynthesis (Steinmüller and Tevini 1985, Barnes *et al.* 1994, 1996, Gordon *et al.* 1998a), and a trend toward shorter chain lengths as a response to UV-B has been documented in cucumber (Tevini & Steinmüller 1987), bean, barley (Steinmüller & Tevini 1985), tobacco (Barnes *et al.* 1994, 1996, Barnes & Cardoso-Vilhena 1996) and pea (*Pisum sativum*) (Gonzalez *et al.* 1996). In conifers, the mechanism may work differently, because at least in spruces no changes in the short- to long-chain-length wax were detected (Gordon *et al.* 1998a), although the result is from chamber experiment.

## 5.2. UV-absorbing compounds

### 5.2.1. Responses to supplemental UV-B

In the field experiment with mature Scots pines the amount of methanol-extractable UV-absorbing compounds was not affected by supplemental UV-B during the first and second growing seasons (IV). During the third growing season, the amount of these compounds was significantly or slightly elevated in the ambient treatment compared to UV-B (VI). These compounds are synthesised only if necessary and required for protection (Mohr & Drumm-Herrel 1983), and because field-grown plants have higher levels of UV-absorbing compounds compared to controlled environments, supplemental UV-B radiation generally induces only scant additional accumulation under field conditions (Dillenburg *et al.* 1995). In field experiments with loblolly pine (Sullivan *et al.* 1996) and two Mediterranean pines (*Pinus pinea*, *Pinus halepensis*) (Petropoulou *et al.* 1995), the amount of UV-absorbing compounds was unaffected after one growing season of UV-B exposure. Still, the even lower amount of UV-absorbing compounds in the present UV-B treatment compared to the ambient treatment was unexpected. The ratio UV-A/UV-B was higher in the ambient treatment compared to the UV-B treatment, and higher levels of UV-A and PAR might stimulate screening compound production at lower or moderate UV-B levels (Day & Demchik 1996), especially in low visible light (Caldwell *et al.* 1994, Middleton & Teramura 1993). However, it is unlikely that this would be notable in the field conditions (Dillenburg *et al.* 1995).

### 5.2.2. Cumulative effects

The lower amount of UV-absorbing compounds in the UV-B treated needles during the third year of supplemental UV-B might be due to the already inhibited synthesis of UV-protective pigments (Flint *et al.* 1985, Deckmyn *et al.* 1994, Sullivan *et al.* 1996), indicating cumulative effects of UV-B radiation (Sullivan & Teramura 1992). The fact that the inhibited pigment synthesis was observed in all needle age classes suggests that the information of the earlier year's UV-B exposure is carried to the new, developing needles.

Cumulative effects might therefore also be observed in the new needles if the tree itself has been exposed to UV-B for several years. This theory is supported by the glutathione results from the same experiment (Laakso, unpublished data). The author observed that the current-year needles had lower total glutathione concentrations under supplemental UV-B treatment than under ambient treatment throughout the season, suggesting that the antioxidant protection mechanism against enhanced UV-B radiation was disturbed after the third year of experiment (Laakso, unpublished data), whereas no UV-B-induced changes in glutathione status were observed after the first year of experiment (Laakso *et al.* 1998).

Cumulative effects were expected to be found in the older needles, which developed during the experiment and received supplemental UV-B for several years. In fact, the c+2 needles collected in July had a 56% higher proportion of stress-induced oxidized glutathione (GSSG %) under supplemental UV-B than in the ambient treatment (Laakso, unpublished data), and during the second growing season's exposure there was a slight trend towards an increasing amount of UV-absorbing compounds in the older needles (11- and 14-month-old needles) (IV). At the end of the third growing season, the c+1 and c+2 needles contained high amounts of UV-absorbing compounds, but no differences were observed between the treatments (VI). However, these previous-year needles were only analysed once at the end of September, when the UV-B-doses were already low.

### ***5.2.3. Changes in the amount of UV-absorbing compounds between seasons and/or needle ages***

It has been suggested that young, still developing needles are more sensitive to UV-B because of their lower amount of UV-absorbing compounds and therefore weaker screening ability (DeLucia *et al.* 1992, Day *et al.* 1992, 1996, Naidu *et al.* 1993). However, in Norway spruce the enzyme involved in the formation of kaempferol 3-O-glucoside was already present in the youngest needles at the beginning of May (Strack *et al.* 1989). In the present study, too, an elevated amount of UV-absorbing compounds was already observed in three-day-old needles (still enclosed their bud scales), where no tubular waxes or chloroform-soluble waxes could yet be detected (VI). These results indicate that secondary metabolites provide protection for young tissues, becoming less abundant and important as the tissue ages and other, non-chemical protective mechanisms may develop, as was concluded by Bennett and Wallsgrave (1994). High amounts of UV-absorbing compounds were also detected in the oldest needles (c+2), where the amount of waxes was already decreased and the wax structure eroded. It may therefore be concluded that Scots pines are well protected against increased UV-B throughout the needle development stages, and only the defence mechanism may vary.

Recently, Fischbach *et al.* (1999) observed the UV screening pigments in Norway spruce needles in UV-B field cabinet experiments during two years, and part of the differences in responses were possibly due to the climatic differences between these two years, which was probably also the case in our three growing seasons' experiment. For example, the higher amount of UV-absorbing compounds in the needles collected in August 1997 could be explained by the warmer and sunnier weather compared to August 1998

(Table 2, Fig. 3). Stephanou and Manetas (1997a,b) also observed a higher amount of UV-absorbing compounds in the sunny and warm summertime compared to the rainy autumn, and even changes in the weather conditions during one day may cause alterations in the amount of UV-absorbing compounds (Veit *et al.* 1996). Strid and Porra (1992) found more abundant accumulation of UV-absorbing compounds in plants that have had the opportunity to recover than in plants repeatedly exposed to UV-B. This observation might be important, since plants in natural conditions are seldom exposed to continuously high UV-B doses. It is interesting to speculate what this observation could mean for the northern plants, which have the autumn time to recover from the UV-B stress, but are exposed to almost continuous light and UV-B in the summer time, although the UV-B doses are only high in the daytime. The repair mechanisms work ineffectively at low temperatures (Björn *et al.* 1997), and if the synthesis of UV-absorbing compounds is also ineffective in a cold climate, it may be crucial for plants in the northern early spring conditions.

#### ***5.2.4. Individual UV- absorbing compounds***

The maximum absorption peak of methanol extraction was observed at 280 nm, whereafter the absorbances steeply decreased, which is typical of woody species (Day *et al.* 1994). Methanol extraction does not differentiate between individual compounds and may also include several other phenolics besides flavonoids (Lindroth & Pajutee 1987, Sauesty *et al.* 1992), and possibly also some other compounds. The absorption at 310 and 320 nm could indicate some flavone and the maximum absorption at 280 nm could be due to kaempferol (Sharma *et al.* 1998), which, together with prodelfinidin, procyanidin, quercetin, and isorhamnetin, is the most common flavonoid in Scots pine needles (Lauranson-Broyer & Lebreton 1993). Schnitzler *et al.* (1996, 1997) found that the main UV-B-induced compounds in Scots pine cotyledons and primary needles were 3",6"-di-para-coumaroyl-isoquercitrin and 3",6"-di-para-coumaroyl-astragaln, but no information is available from mature Scots pines. Because the responses of individual flavonoids to UV-B radiation may be different (Lavola *et al.* 1997, Fischbach *et al.* 1999), it is possible that the amounts of certain flavonoids also increased significantly in the present study, although the methanol extraction revealed no UV-B-induced changes.

There are also other compounds, such as ferulic acid and other bound phenylpropanoids, which absorb strongly at 260 to 280 nm and are co-polymerized with cutin and lignin in the cell wall, and may play a dominant role in the UV screening of conifer species (DeLucia *et al.*, 1992 and references therein). Because these compounds are released from the cell walls by alkaline hydrolysis and are not methanol-extractable (Strack *et al.* 1988, van de Staaij *et al.* 1995), their role in the protection against UV-B remained unclear in the present study. Investment in these cell-wall-bound compounds, thicker epidermis or other protective mechanisms instead of methanol-extractable UV-absorbing compounds could partly explain the lower amount of UV-absorbing compounds in supplemental UV-B compared to ambient treatment (VI). In Scots pine, UV-B radiation did

not induce the biosynthesis of wall-bound metabolites in cotyledons (Schnitzler *et al.* 1996), but there is no knowledge of their role in the protection in mature trees (Laakso & Huttunen 1998).

### ***5.2.5. Advantages and disadvantages of defence mechanisms***

It is still unknown which protective mechanism is selected in each situation, although it might be concluded that flavonoids are produced when the epidermis itself is not a sufficiently efficient UV-B filter, due to the undeveloped or already eroded wax structure. For example, if the epicuticular waxes of the current year needles have been degraded by air pollutants, the wax structure may be an ineffective barrier against UV-B in the future, because no new waxes are produced after the first year. Furthermore, unlike the UV-absorbing compounds, the hairs or epicuticular wax crystals may also reduce PAR transmission (Cen & Bornman 1990, Bornman & Vogelmann 1991, Krauss *et al.* 1997). According to Lowry *et al.* (1980), the cuticular defences are costly in terms of protection per unit of synthesis and only effective in intense visible radiation. Flavonoids are suggested to be the primary means of attenuating UV-B also because they are synthesized rapidly upon exposure to UV-B (Caldwell *et al.* 1983, Jordan 1996). For example, Scots pine seedlings reacted within 72 hours of UV-B irradiation by accumulation of flavonoids (Schnitzler *et al.* 1993). The production of secondary metabolites is also a significant cost (Bennett & Wallsgrove 1994), but in contrast to terpenoids, for example, the production of phenolic compounds is not especially costly (Gershenzon 1993). Furthermore, the secondary substances might be recycled by the plant and therefore used for its primary metabolism (see Fagerström *et al.* 1987).

It should be kept in mind that the possible changes in the amount or distribution of waxes or UV-absorbing compounds are connected to the other functions of the plant. The altered wax composition may change the reflectance properties of the leaves, but may also reduce transpiration, alter the uptake of aqueous chemicals and affect the responses of herbivores to the plants (Jordan 1996). Changes in secondary chemistry may have important implications for plant herbivores, and the decomposition rates and products of plant litter, change the physiology of plants and possibly influence the ecosystem function (Rozema *et al.* 1997b).



## 6. Conclusions

Enhanced UV-B caused some significant changes in the amount and distribution of needle waxes of Norway spruce seedlings in the short-term greenhouse experiment, whereas no UV-B-induced changes in the needle waxes of Norway spruce and Scots pine seedlings were observed in the long-term greenhouse experiment. The amount and distribution of waxes is determined by several environmental factors, and the responses from the greenhouse experiments cannot therefore be directly extrapolated to nature. The field experiment with mature Scots pines revealed no UV-B-induced changes in the amount of waxes during the third growing season or in WTD during three growing seasons with different weather conditions. The mature pines in the northern conditions have naturally xeromorphic, waxy needle surfaces which probably protect the needles well against increased UV-B radiation without any additional wax production. It is likely that changes in the wax chemistry or some other defence mechanisms are more effective.

Contrary to the earlier speculations, even the three-day-old needles seemed to be well protected against UV-B. The selected protective mechanism is partly dependent on the development stage of the needles, because a high amount of UV-absorbing compounds was observed when no chloroform-soluble waxes or tubular waxes were yet detected (the youngest needles) or the waxes were already eroded (the oldest needles). Supplemental UV-B had no influence on the amount of these compounds during the first and second growing seasons, but during the third season the amount of UV-absorbing compounds was slightly or significantly elevated in the ambient treatment. This could indicate cumulative effects of UV-B radiation in the form of already inhibited pigment synthesis. This observation is remarkable, since it may indicate that these defence mechanisms may not be effective enough in the long term to protect Scots pines against the harmful effects of UV-B. Compared to the earlier experiments, this three-growing-season field study can be considered a long-term experiment, but it is still only a short time in the lifespan of Scots pines, and more obvious responses might be detected even later.

Additional research is still needed to clarify at least the following points:

- the cumulative effects on needles which are exposed to enhanced UV-B for their whole lifespan
- the amount of UV-absorbing compounds and wall-bound pigments and their protective role in the early spring

- the responses to individual flavonoids and wall-bound pigments analysed by HPLC and waxes by gas chromatography-mass spectrometry (Gc-MS)
- increased UV-B in the presence of other stress factors, such as elevated O<sub>3</sub> and CO<sub>2</sub>, increased/decreased temperature, drought, etc.

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