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Birch (*Betula pendula* Roth.) responses to high UV-B radiation

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Responses of European silver birch seedlings to highly enhanced levels of UV-B radiation (14.4 or 22.5 $kJm^{-2}d^{-1}$ $UV\text{-}B_{BE}$) were studied in a glasshouse experiment. Visible symptoms, membrane lipid composition, secondary metabolites, mRNA levels of chalcone synthase (*Chs*), localisation of *Chs* mRNA and leaf structure were analysed. Interveinal chlorosis and asymmetric leaves were induced by UV-B. A significant UV-B -induced increase in 18:2 fatty acid of both glycolipids and phospholipids was detected. UV-B dependent increase in accumulation of a quercetin-3-glycoside and three other compounds, as well as a transient increase in *Chs* mRNA levels are indicative of induced biosynthesis of flavonoids. *In situ* experiments showed that mRNA of *Chs* was mainly found in the upper mesophyll and vascular bundle tissues. Structural observations showed injuries caused by high irradiances. At the light microscopy level there was first a reduction in palisade parenchyma and later a more dense structure of spongy parenchyma cells. Electron microscopy demonstrated an increase in vacuolar membrane whorls and dark bodies, occurrence of lipid material and darkened chloroplast stroma in mesophyll cells. However, results also indicated that *Betula pendula* is capable of inducing defence reactions such as biosynthesis of flavonoids even at very high UV-B radiation.

Introduction

A reduction in the stratospheric ozone layer will result in an increase in short-wave solar ultraviolet-B (UV-B) radiation (Madronich *et al*. 1995). Since UV-B radiation is strongly absorbed by nucleic acids and proteins, in turn disturbing their structure and function, UV-B presents a potential risk for plant growth and physiology. There is increasing evidence of the deleterious effects of UV-B radiation on plants (Rozema *et al*. 1997). Exclusion experiments have shown that even ambient UV-B radiation levels can decrease plant growth (Sharma *et al*. 1991) and alter the optical characteristics of leaves (Tosserams *et al*. 1996).

Numerous studies have demonstrated that relatively high UV-B may induce physiological damage in annual agricultural species (e.g., Fiscus and Booker 1995, Caldwell *et al*. 1995) but fewer studies have been undertaken on hardwood deciduous trees (Tevini and Teramura 1989, Day *et al*. 1994, Dillenburg *et al*. 1995, Newsham *et al*. 1996). There is a large variation in sensitivity to UV-B radiation among species (Krupa and Kickert 1989). European silver birch (*Betula pendula* Roth.) is widely-distributed and the most important deciduous forest species in Finland and only little information about its, or other birch species' UV-B sensitivity, exists (Lavola *et al*. 1997, 1998, Lavola 1998). The aim of this study was to determine generally whether supplemental UV-B radiation would alter the growth, leaf structure, lipid and secondary compound metabolism, and photosynthesis related parameters of birch seedlings in a relatively long-term (40 days) glasshouse experiment.

Material and methods

Plant material and experimental layout

The experiment was carried out in the glasshouses of Kuopio University Botanical Gardens. Two-year-old birch (*Betula pendula*) seedlings used in this experiment originated from Suonenjoki Forest Research Institute Nursery. Dormant birch seedlings were planted in a peat/sand mixture in pots 12 cm in diameter, on March 13 and the UV-B experiment (6 h d^{-1}, square-wave type experiment) was started two days later at the time of bud burst. Seedlings were watered daily and fertilised once a week (0.1% 9-Superex, N-P-K, 19:5:20). The experiment lasted for 40 days. Samples were collected from all treatment groups and also from "controls" that had been exposed to lower UV-B for 7 days in the beginning of the study and then removed from beneath the lamps. These 50 seedlings were located in the same glasshouse and experienced the same conditions (with the exception of UV-B radiation) for the rest of exposure.

Two groups of seedlings received either 14.4 kJ m^{-2} d^{-1} (lower UV-B) or 22.5 kJ m^{-2} d^{-1} UV-B_{BE} (higher UV-B) throughout their development. A third group of seedlings was initially exposed to 14.4 kJ m^{-2} d^{-1} then, after the first leaves were fully expanded, to 22.5 kJ m^{-2} d^{-1} UV-B_{BE} radiation, in order to determine whether mature leaves are sensitive to UV-B radiation. The spectral irradiance at the level of the seedling canopy was either 0.056 (lower UV-B) or 0.088 (higher UV-B) W m^{-2} at wavelength 300 nm. A spectroradiometer (Macam SR 9910) was used daily to check the UV-B irradiance above the birch canopy. Philips TL40/12 lamps were potentiometrically adjusted to achieve the targeted UV-B radiation. UV-C radiation was excluded by a cellulose diacetate filter (cut-off point at 290 nm, thickness 100 μm), which was changed twice weekly. PAR values were measured above the seedlings with a Licor LI-185B photometer and varied between 120 and 1 100 μmol m^{-2} s^{-1} depending on weather conditions, thus resulting in variable UV-B/PAR ratios. Total hours of sunshine (PAR appr. 1 100 μmol m^{-2} s^{-1}) during the experiment were 213.9 h. Only twelve cloudy days (less than 2 h sunshine) were recorded. Each of three treatments had two replicates (separate lamp banks), each replicate consisting of 25 seedlings. Visible symptoms of leaves were recorded throughout the experiment.

Original planned amounts of radiation were 3.2 (equivalent to clear-sky conditions in central Finland in June according to Madronich 1993) and 5 kJ m^{-2} d^{-1} UV-B_{BE} (generalised plant action spectrum, normalised to 300 nm, Caldwell 1971). However, a computation error in the Macam spectroradiometer software calculated incorrect values for the biologically effective radiation. The

actual amounts were thus 4.5 times higher than originally targeted. In effect there were three groups each receiving a variably high UV-B dose plus the above mentioned controls.

All statistical analyses were performed using the analysis of variance procedures in the SPSS (SPSS Inc., Chicago, Ill.) package. Since there were no significant differences between the two replicates and the variances were equal, all data were pooled for further analysis.

Analysis of membrane lipids and lipid peroxidation products

Lipids were extracted from frozen birch leaf tissue (ca. 0.2–0.5 g) collected on days 12 and 26 from UV-B exposed and control seedlings. Pooled samples of fully-expanded leaves from 6–8 trees were ground to a fine powder with liquid nitrogen in a mortar (n = 2). Frozen leaf samples were weighed and boiling isopropanol (5 ml) added. The mixture was boiled for two minutes. After rapid cooling, 45 ml of butylated hydroxy toluene was added to prevent oxidation. Methanol (5 ml) and chloroform (20 ml) were also added, and the mixture was shaken for 90 minutes in ice. The mixture was then filtered (Whatman 2) using a Buchner funnel. In order to remove water soluble contaminants a 'Folch wash' was carried out (Folch *et al*. 1957). Three ml (about 25% of total volume) of 1.1M KCl (8 g in 100 ml water) was added to each sample. Samples were mixed and centrifuged at 3 000 rpm (1 268 g) for 3 minutes. The lower phase was then collected, and 3 ml of 50% aqueous methanol was added to each sample. After centrifugation, the upper phase was removed and the lower phase was dried at 32 °C under a flow of nitrogen gas in a test tube heater and then resuspended in chloroform. The samples were stored at –20 °C.

The separation into neutral lipids, glycolipids and phopholipid classes was achieved using SEP-PAK silica cartridges as previously described (Norman and St. John 1987). The fatty acid methyl esters were prepared directly by boron trifluoride-methanol method (Morrison and Smith 1964) and the fatty acid separation was achieved by gas chromatography (GC) as described in Ryyppö *et al*. (1994).

The level of lipid peroxidation products in the leaves was expressed as thiobarbituric acid reactive metabolites (TBArm) (Buege and Aust 1978). The assay was carried out as described in Zwiazek and Shay (1988) with some modification. Leaf samples (0.1–0.3 g) were ground in boiling methanol (5 ml). After filtering, 1 ml samples were combined with 2 ml of TCA–TBA–HCl reagent (15% w/v TCA, 0.375% w/v TBA, 0.25 N HCl) and heated for 15 minutes in a boiling water bath. After cooling, the precipitate was removed by centrifugation at 2 500 rpm (880 g) for 5 min. The supernatant was adjusted to the same volume in all samples with deionised water and the absorbance was measured at 535 nm against blanks. The values were corrected for unspecific turbidity by subtracting the absorbance at 600 nm.

Analysis of secondary metabolites

The secondary metabolite analysis was carried out from the pooled (six to seven trees per treatment) and frozen leaf material collected on day 40 from both UV-B exposed and control seedlings. Leaf tissues were homogenised in liquid nitrogen, and 80 mg aliquots were extracted with 800 µl methanol at room temperature in the dark for 1 h. The cell debris was removed by centrifugation at 11 000 rpm (Biofuge B, Heraeus Christ) for 10 min. The clear supernatant was stored at –80 °C until analysis by HPLC as earlier described by Rosemann *et al*. (1991). Detection was at 280 nm with a UV/visible diode-array detector (Beckman Model 168) and with a spectrofluorimeter (excitation at 300 nm, emission at 400 nm; Shimadzu Model RF 530). The compounds were identified by the retention time, their diode-array spectra, as well as by chromatography with authentic reference compounds.

RNA extraction and northern analysis

Northern analysis was used to determine the mRNA levels of chalcone synthase (*Chs*) gene of UV-B exposed fully-expanded birch leaves. Pooled samples, each 6 trees per treatment, were

collected on day 12 at 0, 6, 10, 24 and 48 h from the beginning of the daily UV-B exposure (daily radiation time was 6 h thus the 24 and 48 h seedlings were removed from the exposure area after 6 h exposure). RNA was isolated from approx. 1 g of frozen leaves using the procedure of Chang *et al.* (1993). For northern analysis, 10 µg total RNA was fractionated on 1.0% agarose gel in MOPS/formaldehyde buffer and transferred by capillary blotting onto positively charged nylon membrane (Boehringer Mannheim). Prior to hybridisation, membranes were stained with methylene blue to ensure even loading of RNA on each lane. Membranes were prehybridised at 50 °C for 6 h in hybridisation buffer containing 7% SDS, 50% deionised formamide, 5 × SSC, 2% Blocking reagent, 50 mM Na_2PO_4 pH 7.0 and 0.1% N-lauroylsarcosine (the DIG System User's Guide for Filter Hybridization, Boehringer, Mannheim). *Chs* cDNA template (EMBL/Genbank Y11022) (R. Pellinen, unpubl.) was isolated from birch cDNA library (Kiiskinen *et al.* 1997). Double stranded DNA probes were generated with PCR using 1 U of DynaZyme DNA-polymerase (Finnzymes), 1.0 µl dNTP labelling mix (Boehringer Mannheim DIG labelling and detection kit), 2.5 mM $MgCl_2$, 30 pmol polylinker specific oligonucleotide primers and 0.1 µg *Chs* cDNA template. PCR conditions were: 95 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min; 35 cycles. Hybridisation took place overnight at 50 °C. Membranes were washed twice for 5 min in 0.1 × SSC, 0.1% SDS at room temperature and twice 15 min in the same solution at 68 °C. Detection was made according to the Boehringer Mannheim's Guide for filter hybridisation with the exception of doubled blocking time. Chemiluminescent detection was carried out by pipetting CSPD substrate directly onto the membrane. Membrane was incubated at 37 °C for 15 min prior to autoradiography at room temperature.

Microscopy and *in situ* hybridisation

Samples for light and electron microscopy were collected from the youngest fully-expanded leaves or from older mature leaves (from 5–8 seedlings per treatment) on days 12 and 40. A 1.5 mm^2 square piece was cut from the basal area of each leaf with a razor blade under a drop of 2.5% glutaraldehyde in 0.1 M phosphate buffer. Leaf pieces were postfixed in 1% buffered osmium tetroxide solution, dehydrated in ascending ethanol series and embedded in LX 112 epon. Sections for light microscopy (LM) were stained by toluidine blue and examined by a Zeiss light microscope. Ultrathin sections were stained with uranyl acetate and lead citrate and were examined by means of a JEOL 1200EX transmission electron microscope (TEM) operating at 80 kV.

The thickness of the whole leaf, upper and lower epidermis, and palisade and spongy parenchyma were measured from the light micrographs. In addition, point analysis was carried out to determine the relative (percentage) areas of epidermis, palisade and spongy parenchyma and intercellular spaces. At the ultrastructural level, the length and width of chloroplasts, and the number and size of plastoglobuli were measured from the electron micrographs. The changes in chloroplast shape, density of stroma, thylakoid swelling, appearance of mitochondria and the occurrence of cytoplasmic lipid droplets were also recorded.

We used the *in situ* hybridisation technique (Simmons *et al.* 1989) for the localisation of *Chs* mRNA. *In situ* samples were collected from the same leaves as for LM and TEM, but were fixed in 4% paraformaldehyde in 1 × PBS, dehydrated in graded ethanol series and embedded in paraffin after xylene treatment. Cut sections (appr. 15 µm thick) were attached to silane coated slides (SuperFrost Plus, Menzel Glaser). Paraffin was removed by xylene, after which slides were hydrated by descending ethanol series. Proteinase K digestion (1 mg ml^{-1}, 30 min, 37 °C) was followed by prehybridisation (30 min, 42 °C) and then hybridisation (overnight, 42 °C) with ^{35}S-labelled antisense RNA probes of *Chs* and *RbcS*. Antisense *RbcS* was used as a positive control and sense *Chs* as a negative control. Radiolabelled probes were produced with Pharmacia TransProbe T DNA kit. Hybridised slides were washed and handled as follows: 4 × SSC, twice in 2 × SSC, RNase A buffer, RNase A (0.02 mg ml^{-1}, 30 min, 37 °C), twice in 2 × SSC, 1 × SSC, ascending ethanol series and air-drying. Visualisation of resulting RNA hybrids was undertaken by microautoradiography with Kodak NTB 2 film emulsion.

Results

Visible symptoms

The first observed visible symptom, chlorosis near the veins, was present 10 days after the experiment began. Symptoms were most pronounced in the upper part of the trees. However, there were no clear differences between UV-B treatments. In the end of the experiment an asymmetry in leaf appearance was observed in about 5% of the leaves in all the seedlings. The asymmetry of an individual leaf appeared as undevelopment of the other axial half of the leaf blade. The oldest leaves were badly wrinkled and showed a silvery coloured appearance in 15% of the seedlings in the higher UV-B treatment.

Lipids and lipid peroxidation products

A profile of the average leaf fatty acid composition of glycolipids and phospholipids among treatments is presented in Table 1. The main fatty acid of birch glycolipids was linolenic acid (18:3), whereas the main fatty acid of phospholipids was linoleic acid (18:2). There were no treatment specific significant changes after 12 days of UV-B exposure except that the proportion of oleic acid (18:1) in the phospholipid fraction tended to decrease (from 8.2% ± 0.7% to 6.8% ± 0.2%, p = 0.057, data not shown) as compared to the controls. After 26 days of exposure there were significant changes in both glycolipids as well as phospholipids when comparing results from UV-B treatments with those of controls. Linoleic acid increased from 31.7% ± 0.2% to 35.2% ± 0.3% (p = 0.010) at the expense of oleic acid, which decreased from 5.8% ± 0.0% to 3.9% ± 0.1% (p = 0.057), and 16:1 also decreased from 4.9% ± 0.2% to 3.7% ± 0.1% (p = 0.010) in the phospholipid fraction. In the glycolipid fraction similar differences in 16:1 (a decrease from 1.5% ± 0.1% to 1% ± 0.2% (p = 0.071) and linoleic acid (an increase from 4.3% ± 0.2% to 6.1% ± 0.2%) (p = 0.004) were detected and, in addition, linoleic acid content was significantly higher in the longer (6.1% ± 0.2%) than in the shorter high UV-B seedlings group (5.4% ± 0.1%).

There were no significant changes in the lipid peroxidation products measured as TBArm products. The mean value was 0.88 (± 0.19) $A_{532-600}$ g^{-1} FW for all the samples.

Secondary metabolites

There were no major differences in the accumulation of methanol-extractable metabolites between the lower and higher UV-B treatments. However, when elution profiles of methanol extracts from the youngest fully-expanded UV-B exposed leaves were compared to controls, a few differences were observed. It appeared that UV-B exposure increased a quercetin 3-glycoside (elution time 31.2 min), and decreased chlorogenic acid (elution time 21.6 min) and an as yet unassigned flavone derivative (elution time 41.7 min). These changes also occurred in the older leaves except that the UV-B-dependent reduction of the flavone derivative was even more pronounced.

Table 1. Glycolipid and phospholipid fatty acid composition (% of total) and bond index (BI) in *Betula pendula* leaves. Values are means of all treatments (eight pooled samples, each from four to eight trees) sampled 26 days after the beginning of the exposure (SD in parentheses). Bond index was calculated as follows: 16:1 + 18:1 + (2×18:2) + (3×18:3) + 20:1 + 22:1. Arrows in boldface and normal arrows indicate the fatty acids which changed significantly (p < 0.05) or almost significantly (p < 0.1), respectively, due to UV-B exposure compared with the controls.

Fatty acid	Glycolipids (% of total)	Phospholipids (% of total)
12:0	0.15 (0.04)	0.12 (0.02)
14:0	0.37 (0.30)	0.09 (0.02)
16:0	8.03 (0.51)	23.82 (0.43)
16:1	1.16 (0.21)↓	4.03 (0.56)**↓**
17:0	0.28 (0.03)	0.31 (0.05)
18:0	4.07 (0.35)	1.96 (0.16)
18:1	1.71 (0.16)	4.38 (0.96)↓
18:2	5.38 (0.71)↑	33.94 (1.51)**↑**
18:3	77.89 (1.08)	29.56 (0.89)
20:0	0.27 (0.12)	0.32 (0.04)
20:1	0.07 (0.06)	0.41 (0.17)
22:0	0.43 (0.17)	0.55 (0.04)
22:1	0.11 (0.02)	0.25 (0.08)
BI	237.56 (17.23)	156.64 (14.46)

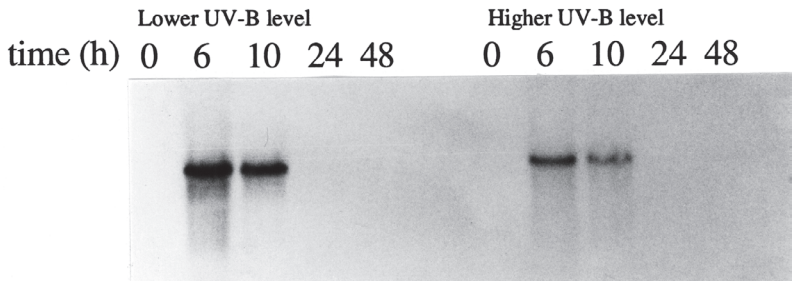

Fig 1. Northern analysis of birch *Chs* 12 days after beginning of the UV-B exposure. mRNA levels of birch *Chs* were clearly induced following UV-B exposure.

When looking at the fluorescence scan (excitation at 300 nm, emission at 400 nm), three metabolites eluting at 14.9, 17.0 and 39.0 min were elevated under increased UV-B irradiance compared to control plants. However, characterisation by on-line diodearray spectroscopy was not successful due to low concentration of the compounds.

Gene transcription

The northern analysis showed that *Chs* mRNA levels increased following UV-B irradiation (Fig. 1). The increase in mRNA level was at its strongest six hours from the beginning of the daily 6-h exposure, and was still evident at ten hours but had disappeared at 24 h. *Chs* mRNA level was substantially stronger in the lower than in the higher UV-B dose exposed seedlings.

Microscopy and *in situ* hybridisation

Anatomical measurements demonstrated a significantly reduced relative palisade parenchyma area, a significantly increased palisade intercellular space (Table 2) and a trend (although not significant) of thickened leaves (Table 3) in the higher UV-B treatment on day 12 (Fig. 2a and b). A significant decrease in spongy intercellular space was

Table 2. Effect of UV-B on relative cross-sectional areas (%) (SD in parentheses) of the various leaf anatomical characters in *Betula pendula* seedlings. Differences in letters denote significant differences ($p < 0.05$) among treatments.

Days of exposure	Treatment	Palisade cells	Palisade intercellular space	Spongy cells	Spongy intercellular space
12	Lower UV-B 12 d	15.7 (4.1) a	5.4 (2.1) a	20.5 (6.5) a	26.8 (5.6) a
	Higher UV-B 12 d	12.3 (3.4) b	7.0 (1.9) b	21.1 (7.3) a	29.8 (5.7) a
40	Lower UV-B 40 d	12.9 (4.8) a	5.8 (2.1) a	12.6 (5.4) a	44.8 (8.0) a
	Higher UV-B 40 d	13.4 (5.1) a	4.1 (1.3) a	18.3 (7.1) a	35.9 (7.0) b
	Lower UV-B 12 d + Higher UV-B 28 d	13.9 (3.4) a	4.8 (2.2) a	19.6 (10.4) a	35.1 (10.6) ab

Table 3. Effect of UV-B on upper and lower epidermis and total leaf thickness of the youngest fully-expanded leaves of two-year old *Betula pendula* seedlings. Values are means of six to eleven seedlings, SD in parentheses. There were no significant differences among treatments.

Days of exposure	Treatment	Leaf thickness (μm)	Upper epidermis thickness (μm)	Lower epidermis thickness (μm)
40	Lower UV-B 40 d	137.9 (11.5)	16.4 (8.2)	9.5 (1.8)
	Higher UV-B 40 d	151.4 (25.3)	20.2 (6.0)	11.4 (2.2)
	Lower UV-B 12 d + higher UV-B 28 d	150.3 (21.1)	19.9 (7.7)	11.0 (3.1)

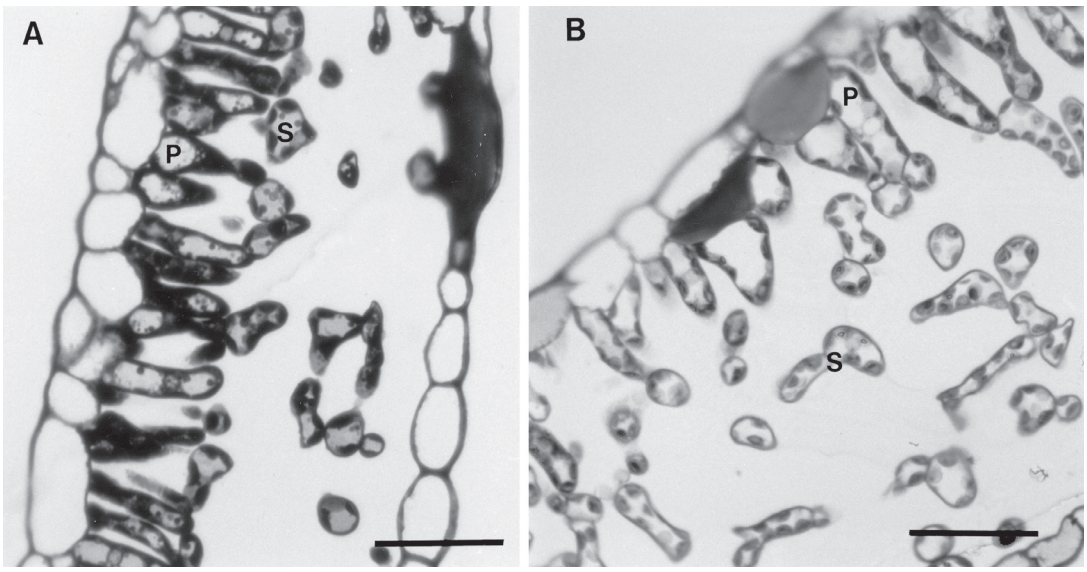

Fig. 2. Light micrographs of lower UV-B (A) and higher UV-B (B) treated birch leaves on day 40. Note the decreased relative area of palisade parenchyma in B. P = palisade parenchyma, S = spongy parenchyma.

detected on the last sampling date.

At the ultrastructural level (Table 4), central vacuoles contained numerous membrane whorls. In addition, a number of dark bodies were observed (Fig. 3a and b). The dark bodies were evident particularly in the central vacuole, where they were associated with membrane whorls, but occasionally appeared also in the chloroplasts of both palisade and spongy mesophyll of all samples. Dark bodies and membrane whorls were most frequent in the higher UV-B treatments and on the last sampling date. There were more of them in the youngest fully-expanded leaves than in the older mature leaves. Lipid bodies were absent in the lower UV-B treatment but existed in small amounts in the higher UV-B treatments. Darkening of chloroplast stroma was induced twice as often in higher UV-B than in the lower UV-B level. Presence of disintegrated mitochondria was already frequent (in more than 50% of the studied cells) by day 12 and increased still further towards the end of the experiment. The percentage of disintegrated mitochondria was highest in the lower UV-B treatment. The size of the plastoglobuli was remarkably larger in the older mature leaves (more than 70% of the studied cells had diameter larger

Table 4. Ultrastructural observations of the presence (%) of dark bodies, membrane whorls, lipids, dark chloroplast stroma and disintegrated mitochondria in the studied palisade parenchyma cells of *Betula pendula* leaves. Higher UV-B treatments have been combined. Number of studied cells was 62–71 on day 12, and 22–30 for the youngest fully-expanded leaves (young) and 8–30 for the older mature leaves (old) on day 40.

Symptom	Day 12		Day 40	
	Lower UV-B Young leaves	Higher UV-B Young leaves	Lower UV-B Young/old leaves	Higher UV-B Young/old leaves
Dark bodies in the central vacuole	13	20	42/11	33/10
Membrane whorls in the central vacuole	65	78	83/75	86/70
Lipid bodies in the cytoplasm	0	3	0/0	16/12
Dark chloroplast stroma	26	46	16/0	36/6
Disintegrated mitochondria	75	53	100/88	76/80

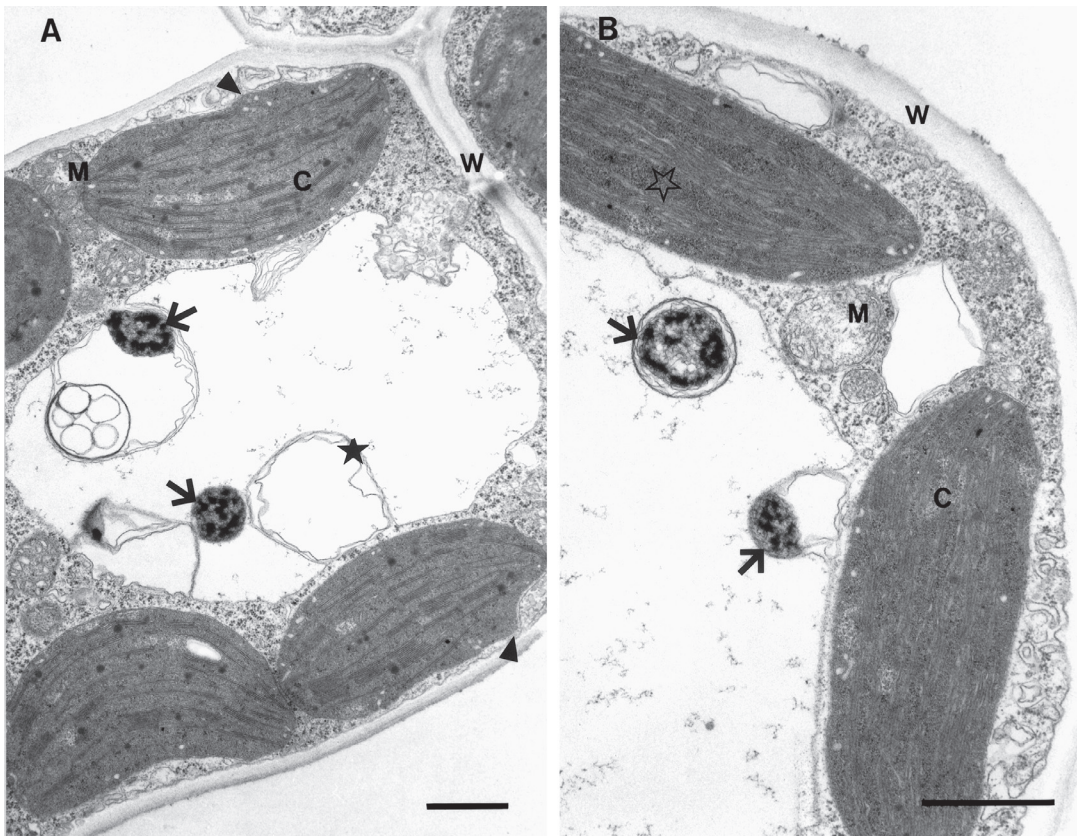

Fig. 3. Electron micrographs of lower UV-B (A) and higher UV-B (B) treated birch leaves. Note the irregular chloroplast shape (arrowhead), and the dark bodies (arrows) and increased number of membrane whorls (asterisk) in the central vacuole. Dark stroma (open asterisk) was typical in the higher UV-B treatment. C = chloroplast, M = mitochondrion, W = cell wall.

than 170 nm) than in the youngest fully-expanded leaves (only 10% of studied cells larger than 170 nm) on day 40 and had increased from day 12 (98% of the studied cells smaller than 90 nm) to day 40. There was a slight trend towards larger plastoglobuli in the higher UV-B levels. There were no consistent treatment-specific changes in the plastoglobuli number, chloroplast size and shape or in starch appearance. However, the chloroplast shape was frequently irregular in all UV-B treatments. Thylakoid swelling was rare occurring in 0%–7% of studied lower UV-B cells and in 0%–13% of higher UV-B cells depending on the day and treatment. Ultrastructural changes were parallel in the spongy (data not shown) and palisade parenchyma cells but treatment-specific differences were more pronounced in the palisade parenchyma.

In situ hybridisation experiments showed that majority of *Chs* mRNA was localised to the upper mesophyll tissue in the cytoplasm of palisade parenchyma cells (Fig. 4b) and to the vascular bundle. Some unspecific background signals were evident in the control sections (Fig. 4a).

Discussion

Despite high radiation levels, the results obtained enable us to assess symptoms and survival of birch in extreme circumstances. Results of this experiment showed that birch (*Betula pendula* Roth.) seedlings can induce defence reactions such as flavonoid biosynthesis even at very high UV-B levels. Some of the analyses (i.e. linoleic acid in glycolipids, light microscopy) demonstrated that

the responses are most severe in treatments with the highest UV-B dose. Most pronounced differences between the exposure groups were observed at the beginning of the experiment. This may indicate that the accumulated UV-B doses in the end were so high, for all UV-B treatment groups, that differences could no longer be detected. Visible symptoms in birch leaves were recorded in this study and can be explained by the unrealistically high UV-B doses (our doses correspond to 80%–180% increase to UV-B_{BE} doses on the Equator in June). Under realistic UV-B supplements, no visible symptoms in leaves of broad-leaved trees have been reported (Dillenburg *et al.* 1995, Newsham *et al.* 1996, Sullivan *et al.* 1994, 1996, Zeuthen *et al.* 1997).

Light microscopical observations showed first a significant thinning (i.e., reduction in the relative area and increase in the intercellular space) of palisade cells (on day 12) and later (on day 40) a significant reduction in the spongy intercellular space and a trend to an increased relative spongy parenchyma area. This suggests that the higher UV-B exposure first resulted in death of some palisade cells and then, in turn, increased exposure of spongy cells to UV radiation forced the spongy cells to form a more dense protective layer. Higher UV-B dose also caused a trend, though not significant, towards an increased thickness of leaves as well as the upper and lower epidermis. Thickening of leaves is a common symptom in UV-B exposed plants (Sullivan *et al.* 1996, Rozema *et al.* 1997).

At the ultrastructural level, numerous abnormal membrane whorls and dark vacuolar bodies were detected in the central vacuoles of both the palisade and spongy parenchyma cells. We associate these structures with secondary metabolite related defence reactions, since similar-looking membrane whorls have been reported in birch stem glands (Raatikainen *et al.* 1992) where high quantities of phenolic compounds are localised. The presence, though in lower amounts, of these structures in the lower UV-B treatment also can be interpreted as a result of the high radiation levels used in this study. This also explains the high percentage (53%–100%) of disintegrated mitochondria in the studied cells. At higher UV-B, the observed lipid accumulations may indicate accelerated cell senescence (Wulff *et al.* 1996) and dark

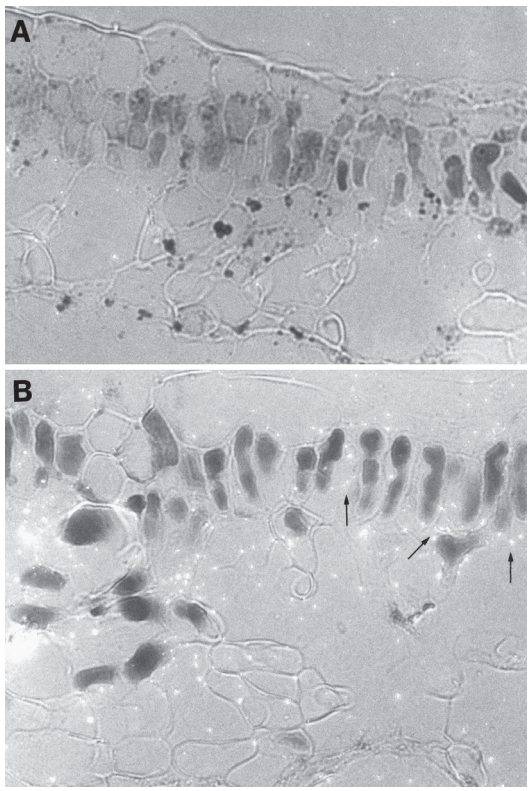

Fig. 4. *In situ* localization of *Chs* mRNA in leaves of UV-B -irradiated birch seedlings. Leaf sections were hybridised with either sense RNA (negative control) (A) or ^{35}S-labelled antisense RNA (B). Note the abundance of *Chs* mRNA in the upper mesophyll tissue (arrows) (B).

stroma suggests perturbation of Rubisco or some other chloroplast protein metabolism.

In glycolipids**,** which represent chloroplast lipids**,** we observed that the higher the UV-B dose the higher the 18:2 levels were. Thus we assume that desaturation to 18:2 was enhanced in the chloroplasts. The 18:1 content was not affected, possibly due to high turnover-rate of 18:1. The rise in 18:2 level can be seen as an adaptation of membranes to high level UV-B, since long-chain polyunsaturated fatty acids are known to be important for thylakoid membrane organisation, composition and function (Hugly *et al.* 1989). In the phospholipid fraction, 16:0, 18:2 and 18:3 comprised more than 80% of total fatty acids. This is similar to phospholipids of pine needle plasma membranes (Anttonen *et al.* 1996). The significant increase of 18:2 in phospholipids in all UV-

B treatments match well with the decrease in 18:1 and demonstrates increased desaturation of 18:1 to 18:2 in the lipid fraction. Alternatively, the elevated levels of 18:2 in phospholipids may be associated with the enhanced synthesis of membranes, the phenomenon which was seen in the ultrastructure as an increased number of membrane whorls. Earlier studies on bean, barley, corn (Tevini *et al.* 1981), cucumber (Kramer *et al.* 1991) and pear (Predieri *et al.* 1995) have suggested that glycolipids — not phospholipids — are the targets of UV-B. In our study, the observed changes in phospholipids suggest that other cellular membranes besides chloroplasts are also affected in the highly elevated UV-B radiation.

Our results do not support the hypothesis of UV-B-induced increase in lipid peroxidation (Kramer *et al.* 1991, Takeuchi *et al.* 1995) since we did not observe any accumulation of MDA or decrease in unsaturated/saturated fatty acid ratios. On the other hand, absence of peroxidation in our experiment may be explained by different test conditions, especially much higher UV-B doses.

Flavonoids and other phenolic substances absorb UV-B radiation and are therefore capable of protecting plants against damage by enhanced UV-B (e.g., Li *et al.* 1993, Middleton and Teramura 1993, Reuber *et al.* 1996). The observed UV-B treatment dependent accumulation of a quercetin 3-glycoside and three compounds observed in the fluorescence scan, as well as a transient increase in *Chs* mRNA levels are indicative of induced biosynthesis of flavonoids. Similar increase of flavonol glycosides in needles of Scots pine has been demonstrated (Schnitzler *et al.* 1997) and more recently in birch leaves (Julkunen-Tiitto *et al.* 1997, Lavola *et al.* 1997, 1998, Lavola 1998). In contrast to earlier studies with Silver birch where chlorogenic acid has either been induced (Lavola *et al.* 1997) or remained unaffected (Lavola 1998, Lavola *et al.* 1998) by UV-B radiation we found a decrease in the levels of this compound. This may be related to the possibly accelerated senescence in our seedlings since Lavola *et al.* (1997) observed a significant age-dependent decrease in chlorogenic acid.

Levels of mRNA of birch *Chs* gene were transiently induced following daily UV-B exposure. Chalcone synthase is a key regulatory enzyme in the phenylpropanoid pathway leading to biosynthesis of UV-protective flavonoid pigments (Jordan *et al.* 1994, Christie and Jenkins 1996) and may be useful as a marker of UV-induced defence reactions. Induction in *Chs* transcripts has frequently been observed following supplemental UV-B radiation (e.g., Strid 1993, Jordan *et al.* 1994). Induced mRNA level of *Chs* was detected in our experiment after 12 days of UV-B (6 h day^{-1}) exposure suggesting that new induction occurs on a daily basis. A similar kind of transient induction of *Chs* transcripts was observed in *Pisum sativum* (Jordan *et al.* 1994, Mackerness *et al.* 1997). Interestingly however, the induction in our study was stronger in the lower UV-B dose exposure. This may indicate that the higher UV-B dose had caused substantial DNA damage (e.g., pyrimidine dimerisation) and therefore the transcription of *Chs* would have been partially inhibited. The observed decrease in the total protein concentration (data not shown) supports the idea of a disturbed protein synthesis in the higher UV-B dose seedlings.

In situ hybridization experiments revealed that *Chs* mRNA in birch leaves was mainly localized in the upper mesophyll tissue. This supports experiments with oats, where chalcone synthase and other enzymes of flavonoid biosynthesis were located in the mesophyll (Knogge and Weissenböck 1986). In Scots pine too, the *Chs* transcripts have also been associated with other than epidermal tissues of the needle (Schnitzler *et al.* 1996) though the attenuation of UV-B radiation is primarily assumed to be a result of accumulation of flavonoids and other phenolic compounds in the epidermis (Day *et al.* 1992, Schnitzler *et al.* 1996). These results suggest that at least part of the flavonoids are not synthesised in the epidermis but are transported there.

The PAR levels in this glasshouse experiment were much affected by the prevailing weather conditions and certainly were not as high as in natural birch stands during the growing season. There is much evidence that low PAR levels exaggerate UV-B induced effects (e.g., Cen and Bornman 1990, Caldwell *et al.* 1994) and therefore our results may actually overestimate birch responses to high UV-B radiation. Though the structural observations showed injuries caused by high irradiances it seems likely that *Betula pendula* can induce defence reactions such as biosynthesis of flavonoids even at very high UV-B levels.

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