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Effect of the temperature and the exclusion of UVB radiation on the phenolics and iridoids in *Menyanthes trifoliata* L. leaves in the subarctic

Françoise Martz [a,b], Minna Turunen [a,*], Riitta Julkunen-Tiitto [c], Kaisa Lakkala [d], Marja-Liisa Sutinen [b,e]

[a] Arctic Centre, University of Lapland, POB 122, FI-96101 Rovaniemi, Finland
[b] Finnish Forest Research Institute, Rovaniemi Research Unit, POB 16, FI-96301 Rovaniemi, Finland
[c] Natural Product Research Laboratories, Faculty of Biosciences, University of Joensuu, POB 111, FI-80101 Joensuu, Finland
[d] Arctic Research Centre, Finnish Meteorological Institute (FMI-ARC), Tähteläntie, 62, FI-99600 Sodankylä, Finland
[e] Department of Biology, University of Oulu, POB 3000, FI-90014 University of Oulu, Finland

This study shows that exclusion of UVB radiation modified the content of flavonols and iridoids but not chlorogenic acids in leaves of Menyanthes trifoliata *in the subarctic.*

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ABSTRACT

The long-term effects of UVB exclusion and temperature on the methanol extractable (ME) phenolics (flavonoids, phenolic acids) and iridoids of *Menyanthes trifoliata* L. (*Mt*) leaves were studied in northern Finland (68°N) using wooden frames covered with filters for UVB exclusion (polyester filter), control (cellulose acetate filter) and ambient (no filter) conditions. Analysis of ambient plots showed no effect of the daily mean temperature ($2\sigma = 1.58$ °C) on the leaf ME compound content and composition, but minimum temperatures decreased the flavonol content. UVB exclusion did not affect the total ME compound content but significantly decreased the proportion of flavonols concomitantly with an increase in iridoids. Due to its high iridoid content, *Mt* appears as an interesting model plant for studying the iridoid biosynthesis and its regulation under stress conditions.

1. Introduction

Menyanthes trifoliata L. (*Mt*) (buckbean) is a perennial aquatic or wetland plant species with a circumpolar distribution from 40°N to the Arctic Circle. It grows north of the Arctic Circle in Scandinavia, Greenland, Alaska and Siberia (Hewett, 1964). The growth habitats of *Mt* include wet bogs, mires, marshes, and the shallow margins of lakes, ponds and watercourses. *Mt* has decreased in range in some regions mainly as a result of the large-scale drainage of wetlands and has been listed as a protected or threatened species in some European countries (Lange, 1998).

Mt has had many historical uses as an emergency and supplementary food, medicine and cattle forage for peoples of the circumpolar north (Jonsson and Jonsson, 1980). In traditional and folk medicine *Mt* leaves are used to treat loss of appetite and dyspeptic complaints, to treat colds, fevers, rheumatism, liver ailments, worms and skin disorders, as an astringent to stop bleeding, and as a remedy against scurvy and other diseases. It is also used as an ingredient in cholagogues, geriatric medicines and anti-inflammatory and antirheumatic formulas (Bisset, 1994; Huang et al., 1995; Jonsson and Jonsson, 1980; Tunón and Bohlin, 1995). *Mt* is a preferred forage of reindeer and elks in Northern Fennoscandia, and caribou and moose in Alaska, USA, with leaves and flowers selected during the snowless time and roots during the winter (Staaland and White, 2001; Warenberg et al., 1997).

The chemical and biological properties of *Mt* have been widely studied (Adamczyk et al., 1990; Battersby et al., 1968a; Ciaceri, 1972; Huang et al., 1995; Junior, 1989; Krebs and Matern, 1957, 1958; Mel'chakova and Kharitonova, 1977). Leaves of *Mt* contain different types of phenolics such as coumarins (e.g., scopoletin), flavonols (rutin, hyperin, trifolioside) and iridoids (loganin, menthiafolin, foliamenthin). Iridoids are water-soluble cyclic monoterpenes that are found in various plant species and are important in defense reactions against insects and mammals (Choi et al., 2004; Murata et al., 2008).

The forecasts for Scandinavia indicate a 2–3 °C increase in mean annual temperature by the late 21st century. Increased concentrations of greenhouse gases will warm the troposphere but cool the stratosphere, leading to more severe ozone depletion and increased UVB radiation (Taalas et al., 2000, 2002; ACIA, 2005; Weatherhead

* Corresponding author. Tel.: +358 40 5391182; fax: +358 163412777.
E-mail address: minna.turunen@ulapland.fi (M. Turunen).

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et al., 2005; IPCC, 2007). Enhanced UVB radiation may increase the concentration of phenolics in reindeer forage plants, which are known to decrease the forage quality, food intake and digestibility in ruminants (Arnold, 1985; Duncan and Poppi, 2008). The aim of the present study was to investigate the effects of long-term (5–6 growing seasons) UV exclusion on the total concentration and chemical composition of methanol extractable (ME) compounds in leaves of *Mt*, which is an important summer forage plant of reindeer and elk in Northern Fennoscandia. We studied these effects by conducting a UV exclusion experiment in an oligotrophic fen used as a reindeer summer pasture in northern Finland (68°N). As a consequence of microtopographic variations in the experimental field, the effect of a small temperature difference on the ME compounds of *Mt* leaves was also evaluated.

2. Material and methods

2.1. Experimental design

The UV exclusion experiment was conducted in northern Finland in a remote oligotrophic tall-sedge *Sphagnum* flark fen, in Kaltiovuoma, Vuotso, Sodankylä (263 m a.s.l., 68°10′N, 26°42′E *Sphagnum*) (Fig. 1A). The ground layer of the experimental site was dominated by *Sphagnum* mosses (*Sphagnum lindbergii* Schimp. in Lindb., *Sphagnum jensenii* H. Lindb., *Sphagnum angustifolium* (C. Jens. ex Russ. C. Jens. in Tolf), *Sphagnum balticum* (Russ.) C. Jens.) and *Warnstorfia heinrich-schulzei*. The field layer was characterized by sedges (*Eriophorum russeolum* Fries ex Hartman, *Eriophorum angustifolium* Honckeny, *Carex rotundata* Wahlenb., *Carex rostrata* Stokes, *Carex limosa* L., *Carex magellanica* Lam. subsp. *irrigua*, *Scheuchzeria palustris* L.), herbs (*M. trifoliata* L., *Rubus chamaemorus* L.) and shrubs (*Andromeda polifolia* L., *Betula nana* L., *Vaccinium oxycoccus* L., *Vaccinium microcarpum* (Turcz. ex Rupr.) Hooker fil.). The surface soil (0–30 cm) was characterized by pH 4.2, C(TOC) 41.5%, N 0.64%, C/N 65, NH_4 82.9 mg kg^{-1}, NO_3 <0.2 mg kg^{-1} and ammonium-lactate extracted (i.e., plant-available) nutrients: P 45 mg kg^{-1}, Ca 1.680 mg kg^{-1}, Mg 568 mg kg^{-1} and K 133 mg kg^{-1} (Soppela et al., 2006).

Temperature and precipitation were recorded during 2002–2007, and UV irradiance was measured with a spectrophotometer (Brewer MKII) on roof of a building at the Arctic Research Centre of the Finnish Meteorological Institute (FMI-ARC) in Tähtelä, Sodankylä (67°22′ N, 26°38′ E) (FMI 2002–2007), 100 km south of the remote experimental site (Fig. 2), as described in Martz et al. (2007). The ambient

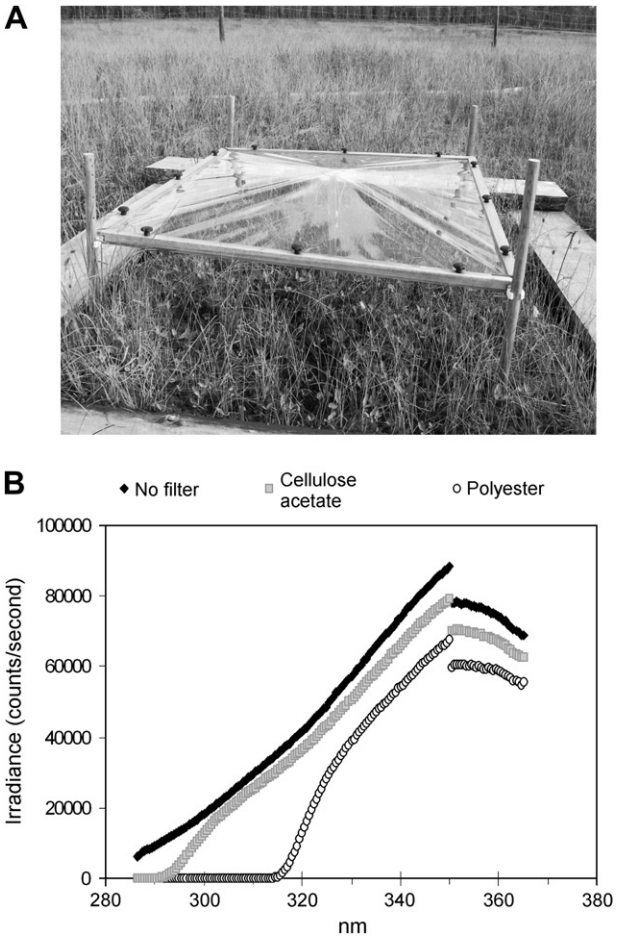

Fig. 1. A UV exclusion plot in Vuotso, northern Finland (end of the growing season) (A) and irradiance under the UV exclusion filters (B).

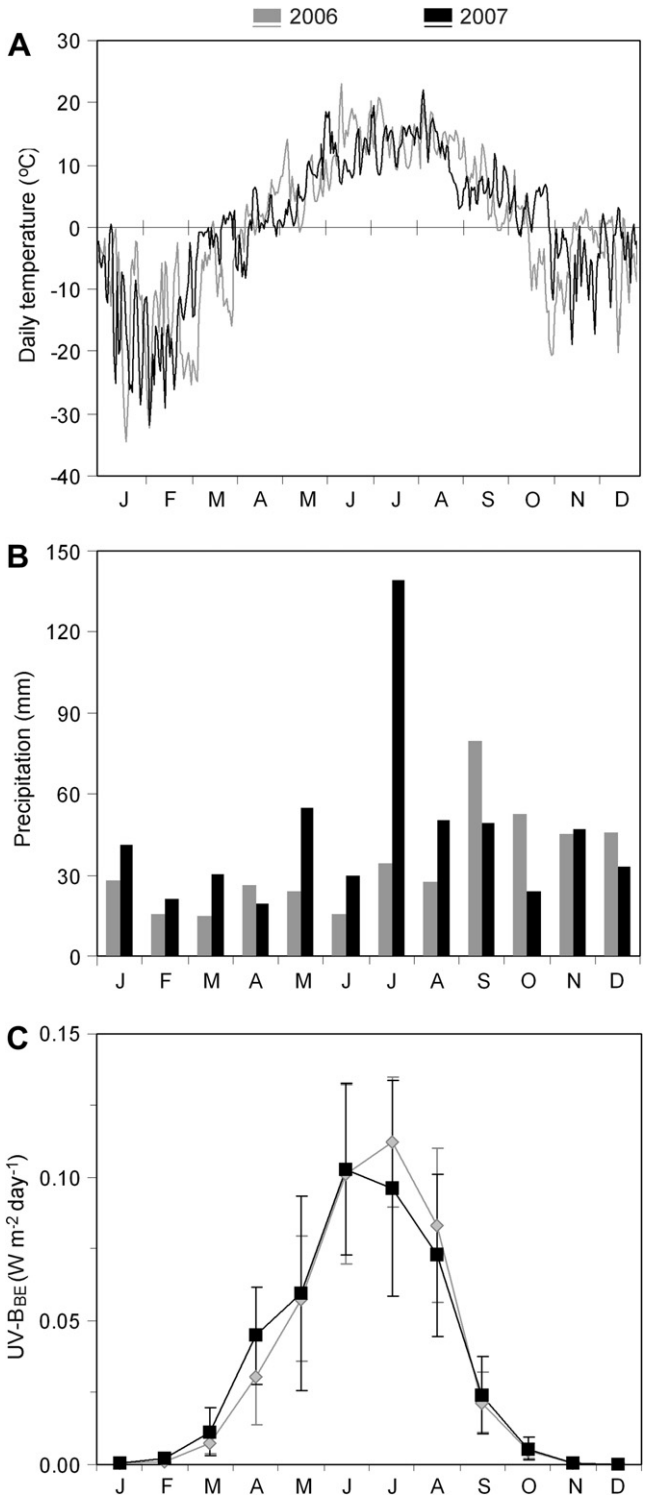

Fig. 2. Mean daily air temperature (A), monthly precipitation (B) and mean monthly UV-B_{BE} (C) in Sodankylä, the closest official weather station, located 100 km south from the experimental site (FMI, 2002–2007). Grey and black lines and bars represent values of 2006 and 2007, respectively. UV-B_{BE}: values are mean ± SD (n = 16–31).

biologically effective UVB (UV-B_{BE}) irradiance was calculated according to Caldwell et al. (1980), and it refers to UV-B_{BE} calculated with the generalized plant action spectrum (Caldwell, 1971). Mean monthly UV-B_{BE} values were calculated from mean daily values.

The experimental site (size about 100 m × 100 m) was fenced off in 2001, because the surrounding area is used as a summer pasture for reindeer of the Lappi Reindeer Herding District. The experiment was arranged in a randomized block design and began in 2002 with three treatments, each conducted in ten plots (altogether 30 plots). On each plot, wooden frames (100 cm × 100 cm) were covered with plastic filters adjusted to 30 cm over a natural fen ecosystem (Fig. 1A). The three kinds of plot were: (1) UVB exclusion treatment plots (-UVB, a clear polyester plastic, 0.125 mm thick, Polifoil, KTA, Ltd., Helsinki, Finland), (2) control plots (CONT, a clear solvent cast acetate film, Clarifoil, Acordis Group, Derby, UK), and (3) ambient plots that lacked filters but had frames (AMB) (Soppela et al., 2006). The polyester transmitted less than 1% of UV radiation below 315 nm, 30% at 320 nm and about 70% above 330 nm, while the acetate filter transmitted light above 290 nm efficiently but less than 1% of UV radiation below 290 nm (Fig. 1B). The absolute UVB dose received by *Mt* shoots at each plot was not measured during 2006–2007. *Mt* shoots were slightly shadowed by taller sedges, the effect being marginal in June, but getting more important towards the end of the season (August). The filters were placed over the frames in early summer before vegetation started to grow, raised during the summer as the plants grew; and removed for the winter. The filters were slightly raised in the center by a central vertical pole so that the rainfall drained to the sides of the plot and not onto the filter. No additional watering was given to the plots because the hydrotopographic level of the flark fen did not change even during the driest summers. To control the warming effect due to the plastic filters, temperature measurements were conducted at every plot with Tiny Talk II Miniature Temperature Dataloggers (range −40 °C to 75 °C; OTLM Software, Orion Components, Ltd., Chichester, UK). The dataloggers were attached to the central pole about 15 cm below the filter and 15 cm above the surface of the *Sphagnum* layer at the beginning of the growing season. Temperatures were recorded every 1.5 h. The experimental periods were as follow: 6 June–5 September 2002 (91 days), 19 May–3 September 2003 (107 days), 17 June–6 September 2004 (81 days), 29 June–9 September 2005 (72 days), 31 May–18 September 2006 (110 days), and 16 June–20 September 2007 (96 days).

2.2. Sampling

Mixed samples of 3–5 individual healthy leaves of *Mt* (possibly some leaves originating from one clone) were collected from each plot at midday on 18 July 2006, 28 June 2007, 26 July 2007 and 23 August 2007. After sampling, the leaves were immediately frozen in liquid nitrogen in the field and stored at −80 °C until processed.

2.3. Extraction and chemical analysis

Frozen samples were ground into fine powder in liquid nitrogen and extracted overnight once in methanol at 4 °C in the dark, centrifuged and stored at 4 °C until analysis. The fresh weight (FW) was measured by weighing the frozen powder added to the methanol for extraction (as an average 0.46 g FW in 10 ml MeOH). The chlorophyll concentrations were measured with a spectrophotometer following the procedure of Porra et al. (1989). 10 μl of the methanolic extracts were analyzed by HPLC (Waters) on a Spherisord ODS-2 column (250 mm × 4.6 mm, 5 μm column with precolumn, Supelco). Samples were eluted from the column by a solvent gradient consisting of Solvent A [0.1% (v/v) H_3PO_4] and Solvent B [100% methanol] at 35 °C with a flow of 1 ml/min. The gradient was in accordance with that used by Keski-Saari and Julkunen-Tiitto (2003). Quantification was carried out at 250 nm with a UV/visible diode-array detector (Waters PDA 996) and selected compounds were further analyzed by HPLC–MS as described in Keski-Saari and Julkunen-Tiitto (2003). Conditions for HPLC/API-ES (positive ions) were as follows: the column was Hypersil Rp C18, ID 2 mm, 10 cm in length; the ES fragmentor voltage was 80–120 eV depending on the compounds; the flow rate was 0.4 ml min^{-1}; and the injected volume was 5 μl. The compounds were identified using retention times, UV spectra and HPLC–MS. The following compounds were used as references: chlorogenic acid (CGA) for all CGA derivatives, caffeic acid, ferulic acid, protocatechuic acid, p-hydroxybenzoic acid, rutin, hyperin for all quercetin derivatives, and kaempferol loganin for all iridoids (all purchased from Sigma–Fluka).

2.4. Statistics

SPSS 15.0 (SPSS, Inc., Chicago, IL, USA) for Windows was used for statistical analysis. After testing the data for their normal distribution, one-way ANOVA was measured to test variation due to treatments. The pairwise comparisons between the treatments were made using Tukey's HSD test (5% and 1% level). To analyze the relationship between the plot temperature (minimum, maximum and mean temperature calculated over 3, 5 or 7 days before sampling) and the ME compound composition, data from each sampling date were centred or normalized to avoid the effect of the seasonal variation in temperature and composition. For each sampling date, the mean values for temperature, total content and composition of ME compounds were calculated. Data from each plot were then normalized to the corresponding mean values: the difference to the mean temperature (centred values) and the ratio to the mean content or composition (normalized values) were further used. The linear relationship between the centred plot temperatures and the normalized compound content or composition was analysed using Pearson correlation analysis.

3. Results

3.1. Environmental conditions

The mean annual temperature in Tähtelä, Sodankylä (100 km south from the remote field site) was 0.70 °C in 2006 and 0.94 °C in 2007, which was warmer than the average annual temperature at the same station during 1971–2000 (Drebs et al., 2002). Summer temperatures were rather similar in both years, but 2007 was characterized by warm months of March and October but a cold month of May (Fig. 2A). The year 2006 was drier than 2007 with 408 mm precipitation compared to 541 mm in 2007 (FMI 2002–2007) (Fig. 2B). The average annual value during 1971–2000 was 509 mm (Drebs et al., 2002). The mean daily biologically effective UVB radiation (UV-B_{BE}) increased from the beginning of March, reached its highest point in June–July (0.112 ± 0.023 Wm^{-2} day^{-1} in 2006 and 0.103 ± 0.030 Wm^{-2} day^{-1} in 2007), and decreased thereafter to its minimum level by the end of October (Fig. 2C).

The mean daily air temperature at the plots was increased due to the presence of filters, with mean values of 14.30 ± 4.29 °C, 15.17 ± 4.48 °C and 14.79 ± 4.29 °C in the AMB (frame only), CONT (cellulose acetate filter) and UVB (polyester filter) plots, respectively, over the period 15 June 2007–31 August 2007 ($n = 78$). Although these values are not statistically different *per se*, this increase in temperature might have biological effects in *Mt* leaves. To further analyze the effect of filters on the temperature, the AMB temperatures were plotted against the difference between the CONT and AMB temperatures (the temperature was recorded every 1.5 h, or 16 times per day for 78 days). The correlation analysis showed that the major effect appeared when AMB temperature exceeded 17 °C (not shown), which happened 45 days between 15 June 2007 and 31 August 2007 (57% of the days). Directly related to this observation, the differences in temperature between the treatments were dependent on the time of the day (Fig. 3). A significant relationship was detected between the AMB plot

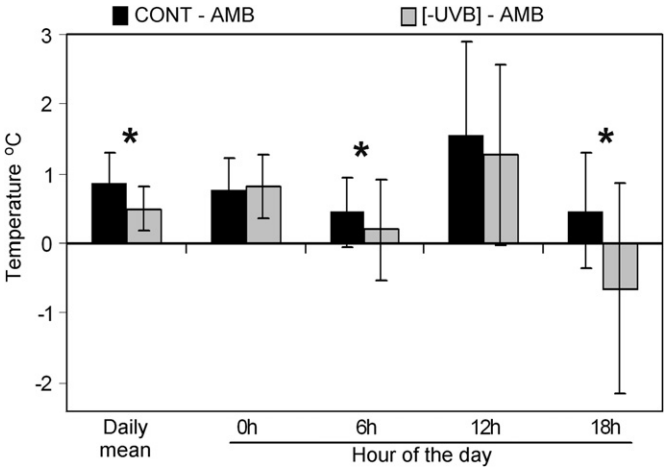

Fig. 3. Warming effect of the two types of plastic filter used on the plots of the UV exclusion experiment. The bars represent the average difference between the CONT and AMB plot temperatures (black bars) and between the [-UVB] and AMB plot temperatures (grey bars), using mean daily temperatures or mean temperatures at 0h, 6h, 12h and 18h during the period 15 June 2007–31 August 2007. Values are mean ± SD ($n = 78$) and similar values significantly different at $p < 0.01$ are indicated by a star.

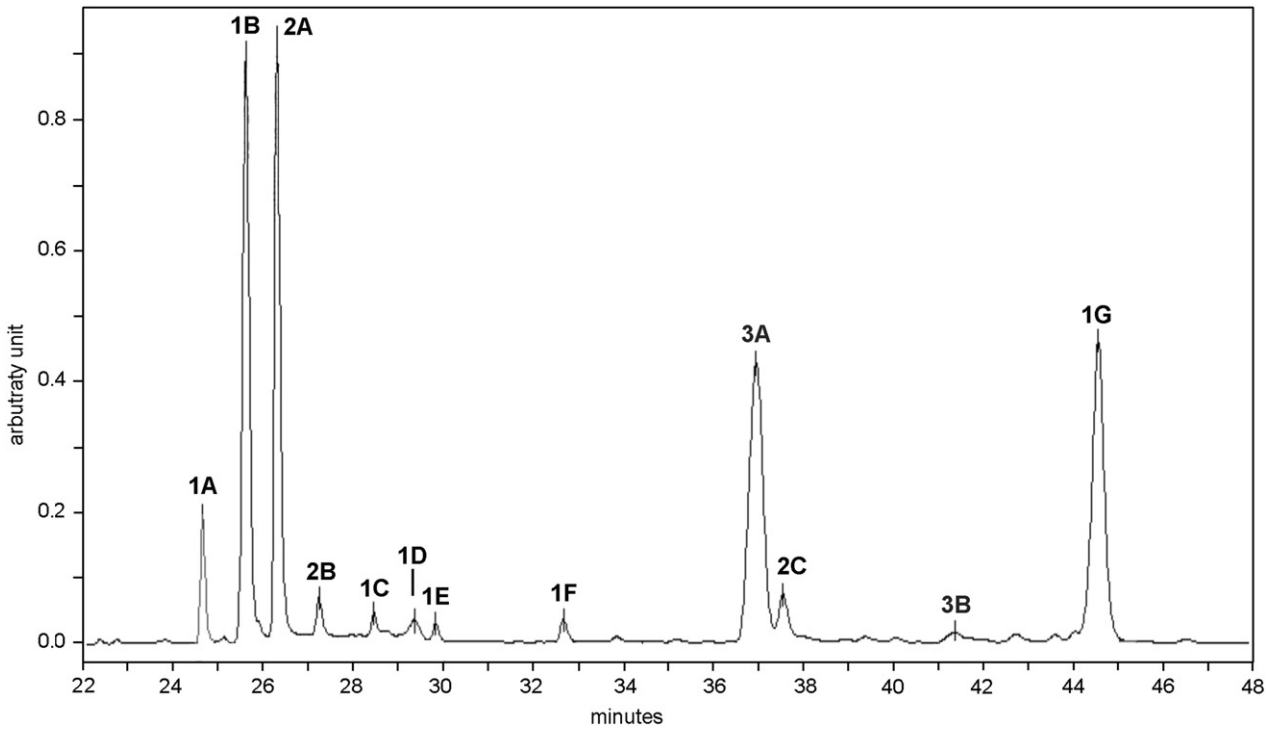

Fig. 4. HPLC-DAD chromatogram at 250 nm of ME compounds of *Mt* leaves.

temperature and the difference between the CONT and AMB plots only at 0 h ($r = -0.443$, $F = 18.586$, $p < 0.001$) and 12 h ($r = 0.636$, $F = 51.469$, $p < 0.001$). This result showed that the highest increase in temperature appeared during the night (shelter effect, negative correlation at 0 h) and particularly at midday (greenhouse effect, positive correlation at 12 h). However, the filter warming effect was lower under the polyester UVB exclusion filter than under the cellulose acetate control filter (differences statistically significant for daily, 6 h and 18 h values at $p = 0.01$) (Fig. 3).

3.2. *ME compounds in* Mt *leaves*

Compounds extracted with methanol were separated by HPLC and quantified at 250 nm. Twelve peaks were separated and the compounds were identified according to their UV spectra and HPLC–MS analysis (Fig. 4, Table 1). Three groups of compounds were identified: iridoids and secoiridoids (further globally called iridoids) (#1 A–G, with two major compounds: secologanate (1B) and dihydrofoliamenthin (1G)), CGA derivatives (#2 A–C, including authentic CGA (2A) and two derivatives (2B and 2C)), and flavonols (#3 A–B: quercetin-glycoside (3A) and kaempferol-glycoside (3B)). At the opposite of CGAs and flavonols, iridoids are cyclic monoterpenes synthesized from the universal terpenoid precursors dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP) mainly via the non-mevalonic acid or MEP pathway (Brechbühler-Bader et al., 1968; Eichinger et al., 1999). Methanol is an efficient solvent for extracting iridoids but may generate artifact compounds due to methylation (Kim et al., 2004; Tomassini et al., 1995). The peak 1F, a loganin methyl derivative, may be such an artifact, but because it is found in low quantities in *Mt* leaves, it does not affect the final results (Table 1). Separation using a different HPLC system coupled with MS analysis showed that the peak 3A in our system corresponded in fact to two different compounds: hyperin (quercetin 3-O-galactoside) and another similar quercetin-glycoside (Table 1). Acid hydrolysis of *Mt* methanolic extracts (2N HCL, 80 °C, 1 h) and comparison with standards

Table 1
Description and amount of the compounds identified on the chromatogram at 250 nm. Amount are mean values ± SD from analysis of AMB *Mt* leaves in 2007 ($n = 30$).

Peak	RT (min)	Compound	Amount (weight %)	λ_{max} (nm)[a]	*m*/*z* (Mr)	Identification[b]
1A	24.3	Loganate	0.9 ± 0.7	235.7	399	I, MS
1B	25.3	Secologanate	25.1 ± 6.1	243.9	397	I, MS
1C	28.6	Loganin	1.2 ± 0.9	236.8	413	I, MS
1D	29.5	Secologanin	2.9 ± 1.5	239.2	411	I, MS
1E	30.0	Secologanin der.	1.0 ± 0.3	233.3	411	I, MS
1F	31.8	Loganin methyl der.	0.4 ± 0.3	235.7	427	I, MS
1G	44.5	Dihydrofoliamentin	18.1 ± 4.0	248	565	I, MS
2A	26.3	CGA	19.4 ± 8.2	216 240 *sh296* 325.7		I
2B	27.2	CGA der.	1.5 ± 0.5	233 319.7		I
2C	37.6	CGA der.	2.5 ± 1.1	214.5 242.7 *sh300*		I
3A	37.0	Hyperin	26.2 ± 5.7	255 *sh266 sh293* 355	487	I, MS
		+ Quercetin glycoside		255 *sh266 sh293* 355	487	MS
3B	41.3	Kaempferol der.	0.9 ± 0.5	264 *sh295* 346		I

[a] Wavelengths of absorption maxima. *sh* = shoulder.
[b] Compound identification method: I: comparison with UV spectra of authentic standards, MS: mass spectra analysis.

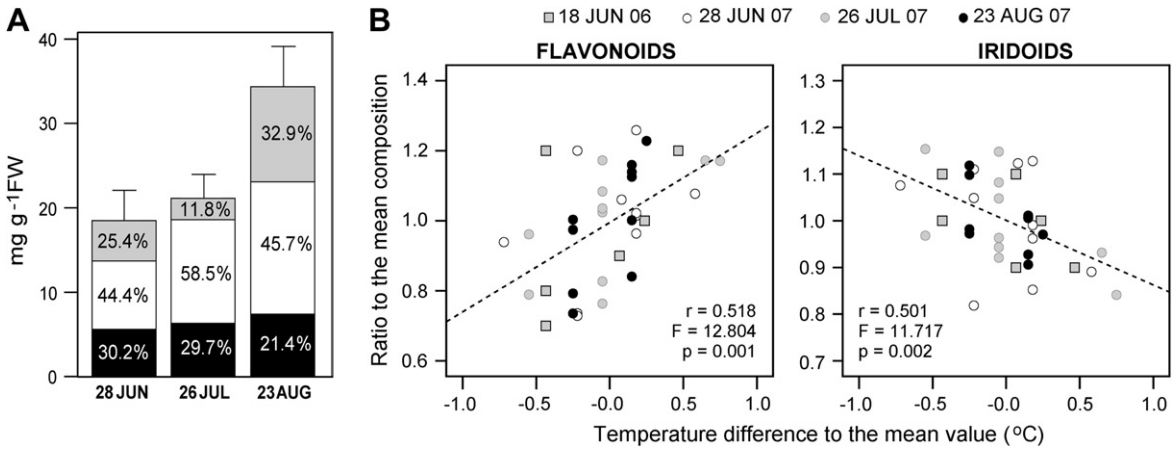

Fig. 5. Effect of the environment on the content and composition of ME compounds of *Mt* leaves under ambient UVB radiation: seasonal changes (A) and significant correlation with the minimum temperature 7 days before sampling (B). The weight % of CGA derivatives (grey), iridoids (white) and flavonols (black) are indicated by bars in A; values are mean ± SD ($n = 10$). Pearson's coefficient r and statistical F values are indicated in B; data are labeled according to the sampling date.

and standard hydrolysis products confirmed the presence of iridoids, CGA (presence of caffeic acid but no ferulic acid), quercetin and kaempferol (not shown).

In AMB samples in 2007, 1 g of fresh *Mt* leaves contained on the average 12.03 ± 3.52 mg (49.6 ± 8.0% of total) of iridoids, 6.19 ± 3.99 mg (23.3 ± 9.4% of total) of CGA derivatives and 6.44 ± 1.78 mg (27.1 ± 6.0% of total) of flavonols, making the total amount of ME compounds to 24.66 ± 7.97 mg ($n = 30$). The total content remained stable during July but increased significantly during August ($F = 49.609$, $p < 0.001$) (Fig. 5A). The amount of the different types of compounds (in mg g^{-1FW}) also varied during the summer with significant changes in CGA derivatives ($F = 114.566$, $p < 0.001$, lowest amount in July), a significant increase in iridoids ($F = 52.099$, $p < 0.001$), but no significant changes in the amount of flavonols ($F = 3.022$, $p = 0.065$).

3.3. Temperature effect

Although not a temperature study *per se*, the microtopographic variations of the experimental field provoked small temperature fluctuations between the plots. This natural variation among the AMB plots was used to estimate the effect of temperature on the leaf composition. The mean temperature and its SD ($n = 10$ plots) at 0 h, 6 h, 12 h and 18 h were calculated every day between 15 June 2007 and 31 August 2007. The average standard deviations were 0.23 °C, 0.49 °C, 1.31 °C and 1.14 °C at 0 h, 6 h, 12 h and 18 h, respectively ($n = 78$ days) and 0.79 °C for all hours included (16 recordings/day, 78 days, $n = 1248$). Detailed plot temperature analysis showed that the differences in standard deviations were due to the plots themselves (all ten AMB plots had a similar temperature pattern throughout the growing season) and not the dataloggers (not shown). CONT plots were not included in the analysis since we cannot rule out the possibility that the cellulose acetate filters had an effect on the leaf composition (Fig. 1A, see below).

To avoid measuring the effect of the season on the temperature or the compound composition, normalized values were used (see 2.4 Statistics). Pearson's correlation coefficients between the minimum, maximum and mean temperature over 3, 5 or 7 days before sampling and the total content or composition of ME compound were calculated. The range of temperature observed in this study (SD = 1.31 °C at 12h and 0.79 °C when all temperature records were included) did not significantly affect the total content of ME compounds, the absolute CGA derivatives or the iridoid

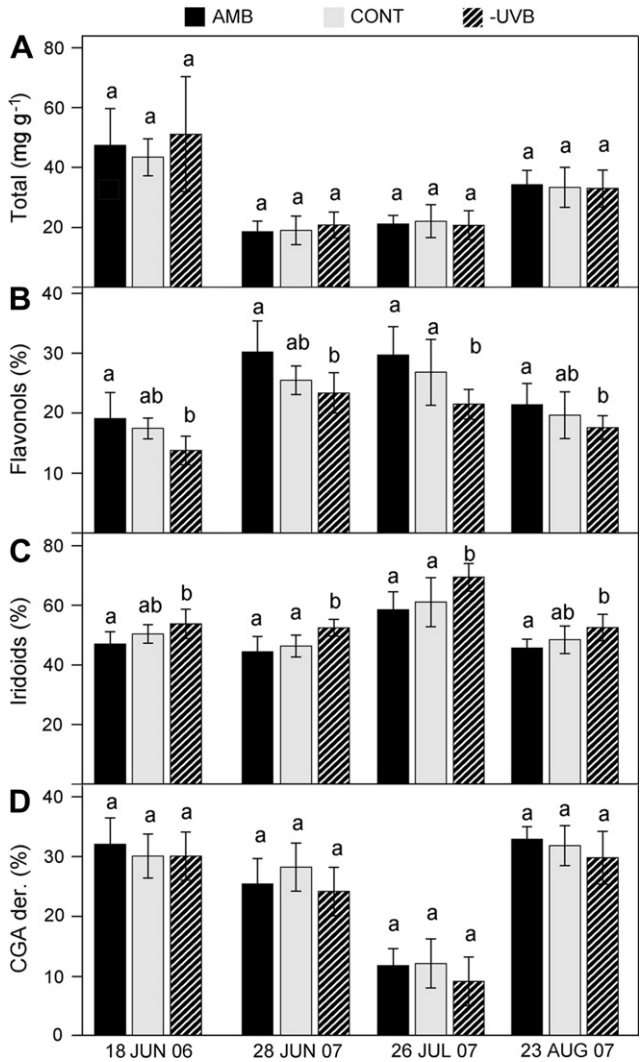

Fig. 6. Total content of ME compounds (mg g^{-1FW}) (A) and relative proportions of flavonols (B), iridoids (C) and CGA derivatives (D) in *Mt* leaves developed under the different conditions. Values are means ± SD ($n = 10$). The letters above the columns indicate significant differences within each sampling time at $p < 0.05$.

amounts (in mg g^{-1FW}, $p > 0.2$, not shown). Nevertheless, the minimum temperature positively affected the amount of flavonols (mg g^{-1FW}) with significant correlations found at 3 and 7 days before sampling ($r = 0.394$, $F = 6.436$, $p = 0.016$ and $r = 0.387$, $F = 6.149$, $p = 0.018$, respectively). Higher correlations ($p < 0.001$) were found between the minimum temperature measured 5 d and 7 d before sampling and the flavonol and iridoid content expressed as % but not the content of CGA derivatives (Fig. 5B). No other significant relationships were found using the minimum temperature before sampling. The minimum temperature recorded during the 7 d before sampling was below zero for each sampling date with −0.60 °C, −2.70 °C, −0.60 °C and −1.40 °C in June 2006, June, July and August 2007, respectively. This result showed that short spell (low freezing temperatures for short period of time) decreased the proportion of flavonols, which consequently also affected the iridoid proportion although this effect is actually due to a decrease in the absolute amount of flavonols.

3.4. Effect of UVB exclusion

Exclusion of UVB radiation did not significantly affect the total concentration of ME compounds in *Mt* leaves but modified its composition (Fig. 6A). Although the amplitude of the effect varied according to the sampling date, UVB exclusion induced a significant decrease in the proportion of flavonols and an increase in that of iridoids (Fig. 6B, C). The concentration of CGA derivatives was not significantly affected by the treatments (Fig. 6D). Similar results were found in both 2006 and 2007.

For both CGA derivatives and flavonols, all individual compounds showed the same pattern as described in Fig. 6. However, the individual iridoids behaved differently under the different treatments, with the secologanate not being affected by UV exclusion whereas the proportion of dihydrofoliamenthin increased under UV exclusion conditions (Fig. 7). No clear trends or significant changes between the treatments were observed with the other iridoids (not shown).

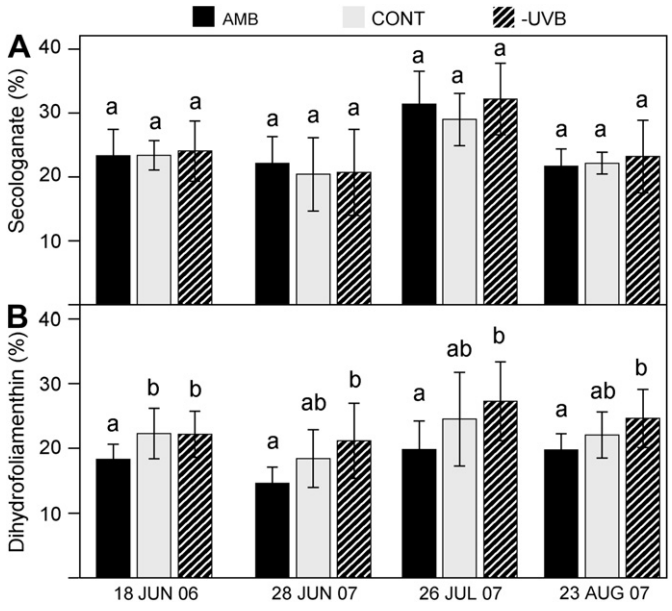

Fig. 7. Proportions of the major iridoids in *Mt* leaves developed under the different irradiance conditions: secologanate (peak 1B) (A) and dihydrofoliamenthin (peak 1G) (B). The letters above the columns indicate significant differences within each sampling time at $p < 0.05$. Values are mean proportions from the total content of ME compounds ± SD ($n = 10$).

4. Discussion

4.1. ME compounds of Mt leaves

In this study, three groups of compounds were detected in *Mt* leaves grown in the subarctic: flavonols, CGAs and iridoids. No protocatechuic acids or coumarins were detected in our samples (Ciaceri, 1972; Swiatek et al., 1986). Flavonols, including quercetin and kaempferol-glycosides have been described previously (Bohm et al., 1986; Krebs and Matern, 1957, 1958; Mel'chakova and Kharitonova, 1977). The HPLC system coupled with MS analysis showed the presence of hyperin and another quercetin-glucoside with an identical UV spectrum. Although rutin was previously reported in *Mt* leaves (Krebs and Matern, 1957; Mel'chakova and Kharitonova, 1977), the second quercetin-glucoside detected in this study is different than rutin according the MS data (Table 1). Iridoids such as loganin, secologanin and dihydrofoliamenthin accounted for about half of all ME compounds in *Mt* leaves. These compounds have been previously reported in *Mt* leaves while folianthin was only detected in rhizomes (Huang et al., 1995; Junior, 1989). Only traces of menthiafolin were detected in our study (RT 46.5 min, not shown). Iridoids and particularly secoiridoids are important precursors for the synthesis of drugs, and *Mt* leaves appear to be an interesting natural source of these compounds (Hallard et al., 1998; Kim et al., 2004) and an interesting plant system in which to analyze the synthesis pathway of iridoids and its regulation under stress conditions.

4.2. Temperature effect

The highest variation in temperature between the AMB plots occurred at midday (1.31 °C at 12 h) and was most probably caused by the microtopographic features and the vegetation patterns of the experimental fen. Although no relationship was found between the daily mean temperature and the total content of ME compounds, the minimum temperature (cold spell lasting only few hours) appeared to affect the flavonol content: low freezing temperatures favored lower content of flavonols which concomitantly increased the content of iridoids. In such high latitudes, temperatures below zero are common, especially in May–June (Drebs et al., 2002). No data was recorded in this study to estimate the role of possible frost-induced leaf damage in flavonol composition, but flavonoids, and particularly flavonols are known for their instability during thawing and chilling (Cannac et al., 2007; Julkunen-Tiitto and Sorsa, 2001). Further analysis will be necessary to explain the effect of frost spell on *Mt* leaf composition in field conditions. Our data did not show any significant effect of the daily mean temperature on the total content or composition of ME compounds, particularly flavonoids, as already described in other plant species (Bilger et al., 2007).

4.3. Influence of UVB

In the present study, *Mt* samples were collected after 5 and 6 growing seasons of UVB exclusion, and no significant difference in the content of total ME compounds between the treated and control samples was measured. Furthermore, no effect on the chlorophyll content or on leaf water content was detected (not shown). These results are in agreement with our previous report from the same field experiment which showed that UVB exclusion over two growing seasons had no effect on the concentration of total phenolics or other measured variables (nitrogen concentration, fibers: lignin, cellulose, *in vitro* digestibility) in *Mt* leaves (Soppela et al., 2006).

Interestingly, significant differences were measured between the CONT and AMB samples. According to the AMB plot analysis, a small increase in the mean day temperature should not have any effect on the leaf content and composition of ME compounds, and a filter warming effect on the minimum temperature would increase the proportion of flavonols with concomitant increase of that of iridoids. However, the opposite trend was observed in the CONT and UVB plots, compared with the AMB plots, which suggests that UV exclusion rather than the temperature increase was responsible for the biochemical changes observed in the CONT and UVB plots.

Our research shows that UVB exclusion resulted in the impoverishment in flavonols with concomitant enrichment of iridoids, which was actually due to variation in the absolute contents of flavonols and iridoids per g of FW (not shown). Flavonols, and particularly quercetin derivatives, have a high antioxidative capacity (Rice-Evans et al., 1997), and the lower content of flavonols under UVB exclusion can be explained by lower UV-induced oxidative stress. Although iridoids may not have a UV-screening effect due to their UV spectrum, their possible regulation under UVB radiation is a completely new result. It is noteworthy that at the opposite of the flavonol individual compounds which were identically affected by UVB exclusion, the different iridoids responded differently to UVB exclusion, and only dihydrofoliamenthin was clearly induced under lower than normal UVB radiation levels.

Iridoid biosynthesis does not have any direct biochemical link with the phenylpropanoid pathway beyond the primary carbon metabolism, with acetyl units required both for the ring A formation of flavonoids and IPP and DMPP synthesis in isoprenoid/monoterpenoid pathway (mevalonic acid pathway) (Bouvier et al., 2005). Crosstalks between the mevanolic acid and the MEP pathways are mainly dependent on species- and physiological-growing conditions (Bouvier et al., 2005). So although loganin synthesis proceeds mainly via the MEP pathway (Battersby et al., 1968b; Eichinger et al., 1999), a weak link between the phenylpropanoid and iridoid synthesis exists but remains speculative to explain the concomitant changes in flavonoids and iridoid content in *Mt* leaves exposed to lower than normal UVB radiation.

Little is known about the regulation of iridoids synthesis in stress conditions (Choi et al., 2004), but interestingly, the sensitivity to oxidative stress of a very early step in the MEP pathway has recently been demonstrated (Rivasseau et al., 2009). The regulation of iridoid pathway under stress conditions *definitely* deserves more attention.

Mt is an important forage plant for reindeer and elks in both summer and winter. Modulation of the secondary compounds of *Mt* leaves, and particularly iridoids known for their bitter taste, may have important consequences on forage plant selection and digestion for reindeer, elk (Duncan and Poppi, 2008) or also the endangered local Cryan's buckmoths (*Hemileuca* spp) larvae which specifically develops on *Mt* leaves (Legge et al., 1996).

5. Conclusion

Field experiment for 5–6 growing seasons showed that 1.58 °C range in the daily mean temperature did not affect *Mt* leaf composition in ME compounds but frosts spells can decrease their flavonol content. Exclusion of UVB radiation had no effect on the total content of ME compounds of *Mt* leaves, but their flavonol content was decreased with concomitant increase in iridoids content, without affecting the content of CGA derivatives. Interestingly, only specific iridoid compounds were affected by exclusion of UVB radiation.

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