Changes in Epicuticular Flavonoids and Photosynthetic Pigments as a Plant Response to UV-B Radiation

Pedro Cuadra[a] and Jeffrey B. Harborne[b,*]

[a] Departamento de Ciencias y Recursos Naturales, Facultad de Ciencias, Universidad de Magallanes, Casilla 113-D, Punta Arenas, Chile
[b] Department of Botany, Plant Sciences Laboratories, The University of Reading, Reading, RG6 6AS, UK

Z. Naturforsch. **51c,** 671–680 (1996); received April 29/June 10, 1996

Gnaphalium vira-vira, Compositae, Epicuticular Flavonoids, Lipophilic Flavones, UV-B Radiation

Treatment of *Gnaphalium vira-vira* plants with UV-B radiation caused changes in plant growth and in plant chemistry. The leaf surface contained two *O*-methylated flavones, araneol and 7-*O*-methylaraneol. HPLC analysis showed that 20 days of UV-B radiation increased the synthesis of 7-*O*-methylaraneol at the expense of araneol. Spectrophotometric analysis of the photosynthetic pigments showed that UV-B radiation also increases the pigment content in treated plants. Another UV alteration is epidermal hair damage, as observed in SEM pictures of treated leaves. This combination of physiological and phytochemical effects may be interpreted as a plant response to UV-B stress.

Introduction

Gnaphalium vira-vira is an annual herb which grows in central Chile predominantly in degraded soils and very exposed valleys. It is broadly distributed within the country, especially between Atacama province (27°S, latitude) in the north and Valdivia province (40°S, latitude) in the south (Reiche, 1905; Hoffmann, 1978). Plants belonging to this genus have been used in folk medicine for a long time. Fresh or boiled preparations are used as strong topical antiseptics, as febrifuges and sudorific agents and in bronchial diseases (Wilhem de Mosbach, 1992; San Martín, 1982).

There are no reports in the literature of detailed phytochemical studies on this plant (Giuffra *et al.*, 1987) but studies carried out on another Chilean *Gnaphalium* species (Urzúa and Cuadra, 1989; 1990) have shown the presence of lipophilic flavonoids with a characteristic pattern based on chrysin. Another interesting feature in *Gnaphalium* is the presence of a mixture of flavonoids and terpenoids in leaf surface extracts.

Epidermal flavonoids and epicuticular waxes are commonly involved in plant environment interactions and their presence has often been suggested as an adaptative response in plants to high levels of sun radiation and high temperatures (Robberecht and Caldwell, 1986; Hoffmann *et al.*, 1983). There is also evidence that they are ecologically important; they may play a role in the defense of plants against herbivorous attack (Karban and Myers, 1989; Harborne, 1991; 1993) or they may act as natural antibiotics (Torrenegra *et al.*, 1989; Cuadra *et al.*, 1994).

Flavonoid constituents in leaf exudates have been involved in ecological interactions (Hedin and Waage, 1986; Urzúa and Cuadra, 1990) and their role in protecting plants against UV-B radiation has also been demonstrated (Tevini *et al.*, 1989; Reuber *et al.*, 1993). They may act primarily as natural screens by absorbing this radiation, but they have an important biological role as efficient hydroxyl radical scavengers (Hussain *et al.*, 1987), which are also produced in plants under UV-B and PAR stress (Neale *et al.*, 1993; Melis, 1992). Other plant pigments such as carotenoids have also been implicated in plant UV-B protection (Rau *et al.*, 1991; Middleton and Teramura, 1994) on the grounds of their physiologically effective quenching properties (Krinsky, 1979; Deming-Adams, 1990).

We therefore set out to examine the role of these epicuticular flavonoids in plant defense and

* Reprint requests to Professor J.B. Harborne. Telefax: +44 (0)1734 753676.

0939–5075/96/0900–0671 $ 06.00

determine which of these two classes of pigments-flavonoids or carotenoids – are involved in the protection of *G. vira-vira* against UV-B radiation.

Materials and Methods

The experiments were conducted from August through mid-November 1994 in an unshaded greenhouse at The University of Reading, Plant Sciences Laboratories. The experimental chamber was built inside the greenhouse. Thus, a metallic framework was designed to hold both treated (T) and control (C) plant groups. UV-absorbing plastic films (CLS grade, Soltech Ltd.) were hung round the treated group to isolate it from the control group. In this way the framework was divided into 2 rectangles (1.98 x 1.16 x 0.97 m). All plants were placed at 0.39 m above ground level.

Plant material

Seeds of *G. vira-vira* (provided by the Botany Section of the National Natural History Museum of Chile) were sown on several plastic trays (0.36 x 0.24 m) containing a John Innes No. 1 plus extra sand compost mixture. After 8 days the seedlings were transplanted into individual plastic pots (26 cm^2), filled with the same compost mixture. Plants were watered every 1–2 days and ambient daytime temperature inside the UV-B chamber was 17–25°C (cold days) and 28–38°C (warm days). All these values were under the maximum temperatures measured inside the greenhouse.

Light sources

Supplemental to greenhouse sunlight (PAR $_{[400-700\ nm]}$:572–1000 μmol m^{-2} sec^{-1}) UV radiation was provided by two fluorescent tubes (Philips TL 20W/12 1220 mm x 1000 mm). According to Adamse and Britz (1992), these tubes were 70 hours pre-burnt in order to keep relatively constant the spectral quality of irradiance output. The UV-B radiation supplementation was obtained by shielding these tubes with a presolarized (7 h) cellulose acetate film (0.075 mm thickness) which cuts off all wavelengths below 280 nm. To avoid UV photodegradation of acetate film, the filters were changed after every 85 h of exposure (Adamse and Britz, 1992; Sisson and Caldwell, 1975).

Radiation measurements

The spectral irradiance levels of plant height below the lamps were measured with a spectroradiometer (Optronics 742, Department of Meteorology, The University of Reading) between 250–500 nm, at 1 nm intervals. The spectroradiometer was calibrated using a NIST traceable 1000 W tungsten filament quartz halogen lamp.

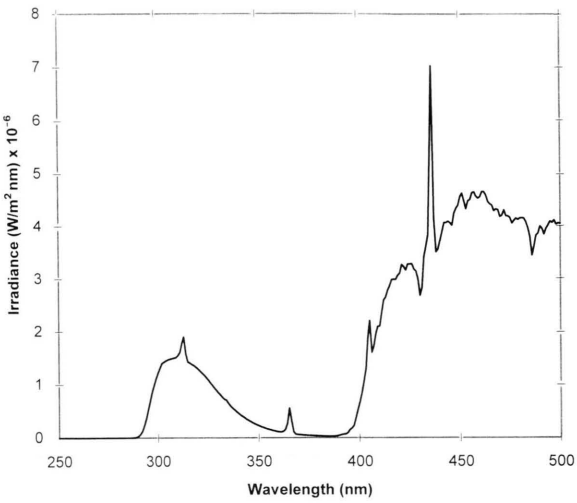


Fig. 1. Spectral distribution of light sources reaching the treated plants. The UV-B irradiance level was achieved by using a combination of 2 fluorescent tubes.

UV treatments

180 plants (65 days old) were distributed in 2 groups inside the experimental chamber. Different UV-B irradiance levels were achieved by varying the distance from plants to lamps. Plants were exposed to 4 and 7 h of daily UV-B irradiation and harvested at different times as different analyses required. Lamps were placed on a mobile rack and hung at 0.70, 0.50 and 0.30 m above the plants. These distances produced a daily fluence rate of 0.23, 0.66 and 1.60 mW m^{-2} of UV-B radiation (280–320 nm) respectively. These values are not normalized or biologically weighed (Coohill, 1989). Pot positions were randomized within each group every 2 days to minimize position effects. Once each week, lamp rack height was adjusted to maintain the UV irradiance levels. After irradiation, the UV-absorbing films were removed in order to give the same light conditions to all plants.

Growth variables

Stem elongation was measured from soil level to the shortest leaf of first stage. Leaf area of adaxial epidermis was measured using a leaf-area meter (Area Measurement System, Delta-T Devices Ltd). Leaves used in area measurements were collected from the top of plants (first and second stages). Each harvest was carried out on 3 different and intact plants. All measurements were carried out weekly.

Epidermal examination

Scanning electron microscope (SEM) examination of leaf epidermis was carried out using a JEOL JSM-T20 scanning microscope, operating at an accelerating voltage of 20 kV. Control and treated leaves were examined after 15 and 45 days of UV-B irradiation. Leaf strips were fixed for 2 h in 5% glutaraldehyde buffered by phosphate (pH 7.3), dehydrated in a graded series of acetone and then dried in a critical-point drier using liquid CO_2. The strips were coated with gold in a vacuum evaporator and then analyzed. Concomitantly with this procedure, leaf samples were frozen in N_2 and then freeze dried (7 h). The specimens were then gold coated and examined. In order to analyze possible effects of UV-B radiation on the hairs of leaves, ordinary pictures were taken of fresh samples using a Wild Photomakroskop M 400 light microscope, equipped with a Wild Photoautomat MPS 55 camera system.

Photosynthetic pigments

Several leaf samples from different stem positions were analyzed. Two leaves from the same stage were weighed, leaf area determined and used in separated pigment analysis. All absorbances were determined for samples in a Cecil CE 1020 monochrome spectrophotometer using quartz cuvettes of 10 mm. The absorbance spectrum were registered in a Philips PO 8720 UV/Vis scanning, fixed 2 nm band with, monochrome spectrophotometer, equipped with a Philips Thermal printer/plotter and a Philips video RGB monitor CM 8533. Total chlorophylls and carotenoids were extracted from individual leaves with 5 ml of DMSO for 12 h at 65°C in the dark, as described in Hiscox and Israeltam (1979). The absorbance was determined at 664, 648 and 470 nm over 1 ml of samples and absorbance spectrum recorded between 250–500 nm. Photosynthetic pigments concentrations were calculated according to equations given in Chapelle and Kim (1992).

Epidermal flavonoids

Fresh whole plants (340 g, aerial parts) were individually extracted by dipping them in *ca.* 300 ml of cold CH_2Cl_2 (analytical Merck) for 10–15 seconds. The extracts were filtered through filter paper (Whatman No.1) and then concentrated until dryness in a rotevaporator at 30°C. The residue was kept at 0°C for further chromatographic analysis.

Chromatographic analysis

Plant extracts were studied by TLC and HPLC techniques. TLC was carried out on silica gel plates (Kieselgel 60, Merck; 0.2 mm), using a petroleum ether (analytical, Merck),(PE: 40–60°)/AcOEt=3:1 (v/v) mixture as eluent system (system A). HPLC analysis was performed in a Waters 600 Multisolvent Delivery System equipped with a programmable Photodiode Array Detector, using a C-18 phenyl packing material column. Aliquots of 20 µl were injected in the column developed by gradient within 25 minutes from 40% A (2% HOAc) to 60% B (MeOH:HOAc:H_2O=18:1:1, v/v). The chromatograms were recorded by a Waters 5200 printer/plotter. Further chromatographic separation was carried out by CC using a glass column (3.0 x 60.0 cm) packed with silica gel (Kieselgel 60; 0.063–0.200 mm; 70–230 mesh ASTM). The column was eluted with increasing polarity mixture of PE:AcOEt (100% – 85% in PE). The collected fractions (5 ml) were monitored by TLC and mixed according to their TLC properties.

HPLC Quantitative analysis

Quantitation of compounds **2** and **3** in both plant groups was performed in a Waters 600 Controller equipped with a Waters 490-E Programmable Multiwavelength Detector using a Spherisorb 55 OD52–6544 packing material column. The column was developed by gradient within 30 minutes from 30% A to 60% B. The chromatograms were recorded in a SP 4270 Integrator which also

automatically calculated the concentrations of standards (**2** and **3**) and samples.

Spectroscopic analysis

The NMR ^1H and ^{13}C spectra were measured using $CDCl_3$ solutions and registered in a Bruker Spectrometer at 400 MHz. COSY, NOESY and H-C long range correlation were also performed in the same spectrometer. EIMS spectra were registered in a Bruker Spectrometer at 90 eV. The UV spectra were registered in an Unicam SP 1805 UV Spectrometer, using methanolic solutions of compounds. The classical shift reagents (Mabry *et al.*, 1970) were used with compounds **2** and **3**. The infra red spectrum (compound **1**) was recorded in a Perkin Elmer 700 IR Spectrometer using KBr discs.

Kaurenoic acid (**1**)

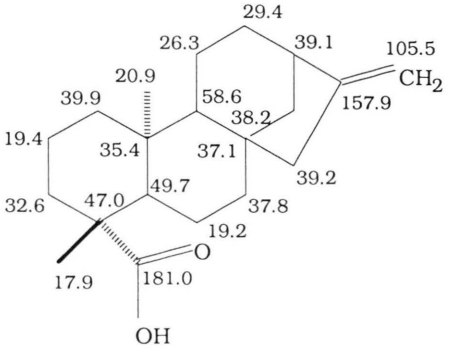
From fractions 5–7 and after crystallization in MeOH (24 h, room temperature) this was obtained as colourless needles (mp 142°C). ^1H-NMR δ=0.97 (3H,s,H-20); 1.27 (3H,s,H-18); 2.65 (1H,br.s., H-13); 4.75 (1H,s,H-17); 4.81 (1H,s,H-17). ^{13}C-NMR δ=183.0 (C-19); 156.1 (C-16); 103.2 (C-17); 57.3 (C-5); 55.4 (C-9); 49.2 (C-15); 44.5 (C8); 44.1 (C-13); 43.9 (C-4); 41.5 (C-7); 40.9 (C-1); 39.93 (C-10); 39.88 (C-14); 33.3 (C-12); 29.2 (C-18); 22.1 (C-6); 19.3 (C-2); 18.7 (C-11); 15.8 (C-20). EIMS *m/z* (rel.int.)=302.2476 (M^+,21.3%, $C_{20}H_{30}O_2$ requires 302.2246); 287 (M^+-15, 19.2%); 259 (M^+-43,26.4%). IRϑcm^{-1}=3500–3350 (b,OH); 2950,870 (s); 1680 (COOH). UVλmax= 208.[+NaOH]:213.

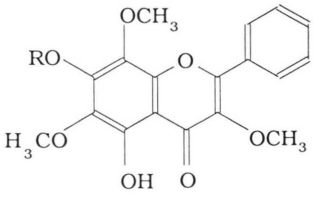
Araneol (5,7-dihydroxy-3,6,8-trimethoxyflavone) (**2**)

Fractions 15–17 underwent successive fractionations using TLC preparative plates (Kieselgel 60; 2.0 mm), eluted with system A and $CHCl_3$:MeOH=96:4 mixtures. Compound **2** appeared as a yellow spot in daylight and as a deep purple spot under UV light (366 nm) and in the presence of NH_3 vapour. It was obtained by scraping off the bands and extracting them with acetone. Further purification was achieved using a Sephadex L-20 column. Crystallization in MeOH (24 h, 0°C) gave yellow needles (mp 151–152°C). EIMS *m/z* (rel.int.)=344.36 (M^+,91.1%; $C_{18}H_{16}O_7$ requires 344.3080); 329 (M^+-15, 100%); 301 (M^+-43,8.8%); 197 (A_1,4.5%); 169 (A_2+1,5.5%); 105 (B_2, 18.2%); 71 (29.0%). UVλmax= 278,326,364,sh.[+$AlCl_3$]: 294,348,424. [+$AlCl_3$+HCl]: 294,344,424. [+NaOH]: 286, 380.[+NaOAc]: 285,380. [+NaOAc+H_3BO_3]: 280,324,364.

7-O-methylaraneol (5-hydroxy-3,6,7,8-tetramethoxyflavone) (**3**)

Fractions 8–10 were fractionated and purified as with **2**. Crystallization in MeOH (24 h, 0°C) yielded orange needles (mp 96°C) which had the same colour properties as **2** in daylight, UV light and NH_3 vapour. EIMS *m/z* (rel.int.)=358.19 (M^+,98.3%; $C_{19}H_{18}O_7$ requires 358.3340); 343 (M^+-15,100%); 315 (M^+-43,4.0%); 229 (A_1,5.4%); 183 (A_2+1,4.7%); 105 (B_2,12.0%); 71 (6.0%). UV-λmax=284,316,365sh. [+$AlCl_3$]: 286,342,430. [+$AlCl_3$+HCl]: 294,342,428. [+NaOH]: 292, 416. [+NaOAc]: 284,318,365. [+NaOAc+H_3BO_3]: 284,316,365.





(2): R = H

(3): R = CH_3

Statistical analysis

Statistical assessment (ANOVA, LSD test) was performed for all measurements using the SAS 6.08 System Statistical Package.

Results and Discussion

Chemistry

From the more polar fractions two highly methylated flavones (**2** and **3**) were isolated. Identification of these metabolites was achieved by using UV and EI-mass spectroscopy. The weak band observed above 350 nm (attributable to band I) indicates an unsubstituted B ring (Cuadra, 1989). EIMS spectra showed the ion molecular peaks at m/z 344 and 358 for a dihydroxytrimethoxyflavone and a monohydroxy tetramethoxyflavone, respectively. The UV spectra after addition of classical shift reagents (Mabry *et al.*, 1970) indicated a 3,5-oxygenated system with a free hydroxyl group at C-5. The bathochromic shift (5 nm) observed after addition of NaOAc in **2** suggests a free hydroxyl group at C-7. This latter effect is absent from **3**, which is 14 mu heavier than **2**. This indicates that methylation at C-7 is the difference between the two compounds. EIMS analysis (RDA fragments) confirmed the presence of unsubstituted B rings (*m/z* 105) and the oxygenation pattern on the A and C rings (*m/z* 197,131 and 211,131). Furthermore, selective methylation of **2** at position C-7 (Markham *et al.*, 1982) gave **3** . Thus, **2** and **3** were identified as 5,7-dihydroxy-3,6,8-trimethoxyflavone (araneol) and 5-hydroxy-3,6,7,8-tetramethoxyflavone (7-O-methylaraneol). They have previously been isolated from *Anaphalis araneosa* (Ali *et al.*, 1979), *Helichrysum decumbens* (Tomás Lorente *et al.*, 1989) and *Helichrysum arenarium* (Hänsel *et al.*, 1967). The third compound, kaurenoic acid (**1**) was readily identified on the basis of IR, MS and NMR spectroscopy (see Methods) and comparison with literature data. It is a well known diterpene acid and has been previously reported from *Gnaphalium undulatum* (Bohlmann and Ziesche, 1980), *G. graveolans* and *G. pellitum* (Torrenegra *et al.*, 1992), *Xylopia aethiopica* (Hasan *et al.*, 1982) and *Helichrysum* ssp. (Bohlmann *et al.*, 1978).

UV-B effects

After 90 days of UV-B irradiation a simple inspection of plants failed to reveal any visible symptoms such as chlorosis or bronze leaf discolouration. However there are other physiological changes which will be discussed below. According to the spectra of the light device (Fig. 1), the triple filter system (glasshouse + UV-cutting filter + UV absorbing film) produced mainly UV-B with low levels of a UV-A component. Hence, all the observed effects on these plants can be attributed to UV-B radiation.

Chemistry

Chromatographic analysis (TLC) of CH_2Cl_2 leaf wash revealed two main compounds with R_f=0.6 and 0.3 (system A). HPLC qualitative analysis using the photodiode array detector confirmed the presence of 2 main UV-absorbing compounds with R_t=12.95 and 16.79 (**2** and **3**, respectively). These flavones were chosen as indicators of UV-B stress. Quantitation of these compounds performed by HPLC analysis revealed a significant variation in production of araneol and its methyl ether in the control plants. Table I shows the concentration of **2** and **3** in non-irradiated plants at different times. Initially both compounds occur in roughly equal amounts ($R_{2/3}$ = 0.9). After a significant increase during the first 20 days araneol concentration decreases gradually (see Table I). In contrast, its methylated derivative decreases considerably during the first 60 days. As a consequence, the final

Table I. Variation[b] of araneol and 7-O-methylaraneol concentrations[a] in non-irradiated plants.

Time (days)	Araneol[c]	7-O-Methylaraneol[d]
0	0.467 ± 0.0266[a]	0.535 ± 0.0254[a]
20	0.567 ± 0.0266[b]	0.419 ± 0.0254[b]
60	0.421 ± 0.0266[a,c]	0.175 ± 0.0254[c]
90	0.372 ± 0.0266[c]	0.108 ± 0.0254[c]

[a]Mean concentration (μg flavonoid/10 μg extract; N=4) and standard error of flavonoid concentration at different times.
[b]Differences between means at different times with the same lower case letter in a column are not significant by T test (LSD=0.0849) controls.
[c]Means after 90 days are statistically different by LSD in ANOVA. DF=3, F=9.74, P < 0.0035.
[d]Means after 90 days are statistically different by LSD in ANOVA. DF=3, F=63.08, P < 0.0001.

ratio (e.g. after 90 days) has increased appreciably ($R_{2/3}$=3.4). Statistical analysis showed that these changes are significant (see Table I). These findings suggest that the *O*-methyltransferase activity is time dependent at this stage in flavonoid biosynthesis and methylation of **2** is taking place before araneol level decreases (20 days) as a natural consequence of plant metabolism.

However, this status appears to be altered by UV-B radiation. Table II shows the mean concentration values of **2** and **3** in control and treated plants. It seems that the methylation process is still active at 20 days of irradiation and produces more 7-*O* methylaraneol than in the control plants. Further irradiation of plants for longer times did not increase this response (data not shown). This lack of correlation in increases in the observed response over longer times of UV exposure was also observed by Tezuka *et al.* (1993). An explanation of this apparent saturation of response in the UV-B photoreceptors will be possible only after a better understanding of the mechanism involved in plant responses to UV-B radiation. Despite flavonoid metabolism being affected by UV-B radiation this modification in flavonoid content is only quantitative because no qualitative differences were detected either by TLC or by HPLC techniques. Regarding the UV spectra of these compounds, it is possible to explain the increased levels of **3** on the grounds that its main absorption peaks at 284 and 316 nm are more closely within the waveband range of UV-B light (280–320 nm) than those of araneol (278 and 326 nm). The extinction coefficient of **3** (log ε 4.31) also shows that the 7-*O* methyl ether has a higher absorption maxima than **2** (log ε 4.11) at shorter wavebands and may therefore be more protective against UV-B radiation than araneol. These findings may be related to those reported in literature about UV-B effects in plant flavonoid content. Early work of Wellman (1971) demostrated that UV light stimulates flavone glycoside synthesis in *Petroselinum hortense* cell suspension cultures. Quantitative effects on flavonoid composition was also found in turnip (*Brassica napus*). Cen *et al.* (1993) reported that quercetin/kaempferol ratio increased strikingly in UV-B irradiated plants. Tevini *et al.* (1981), working on four crop species, found that the flavonoid content increased in barley (*Hordeum vulgare*) and radish (*Raphanus sativus*)

Table II. Effect of UV-B radiation on araneol and 7-O-methylaraneol content[a].

Compound[b]	Control	Treatment	F[c]	P[d]
Araneol	0.567±0.0272[a]	0.560±0.0272[a]	0.03	0.8684ns
7-O-Mearaneol	0.419±0.0033[a']	0.522±0.0033[b']	494.27	0.0002

[a]Mean concentration (μg flavonoid/10 μg extract; N=8) and standard error of flavonoids after 20 days of UV-B exposure. Means followed by different lower case letter in the same row are significantly different at P < 0.005 level as determined by LSD in ANOVA.
[b]Degrees of Freedom=1; F and P (determined in ANOVA) are given for each plant flavonoid by treatment.
[c]F-value was obtained by dividing the mean square for group by the mean square for residual.
[d]Significance level was determined using 4 and 3 DF.ns= no significant.

seedlings by about 50% as a response to the increased UV-B exposure. More evidence about UV B effects on plant flavonoids is found in Mirecki and Teramura (1984), Wellman (1985) and Middleton and Teramura (1993; 1994). These authors show that plants accumulate unidentified UV-B absorbing pigments (reported as absorbance at 300 nm) after irradiation with UV-B light. There are few studies where more specifically increases in individual flavonoids have been reported. Beggs *et al.* (1985) found that isoflavonoid formation occurred as an indicator of UV stress in leaves of *Phaseolus vulgaris*. They showed that coumestrol synthesis is UV-induced and it is linearly dependent on UV-B fluence. This fluence-response relationship was also seen in etiolated radish seedlings exposed to UV-B radiation (Tevini *et.al.*, 1989). These increases in flavonoid concentration are presumably linked to increases in enzymatic activity and in fact, several key enzymes of the flavonoid biosynthetic pathway have also been shown to be specifically induced by UV-B radiation (Wellman, 1975; Schmelzer *et al.*, 1988). Tevini *et al.* (1989) have demonstrated, in leaves of rye (*Secale cereale*) seedlings, that the accumulation of two isovitexin derivatives – exclusively located in the epidermal layers is due to a higher activity of the enzyme phenylalanine ammonia lyase (PAL) and/or higher rates of its biosynthesis.

Plant physiology

Scanning electron microscopic examination of control and treated leaf epidermises showed little

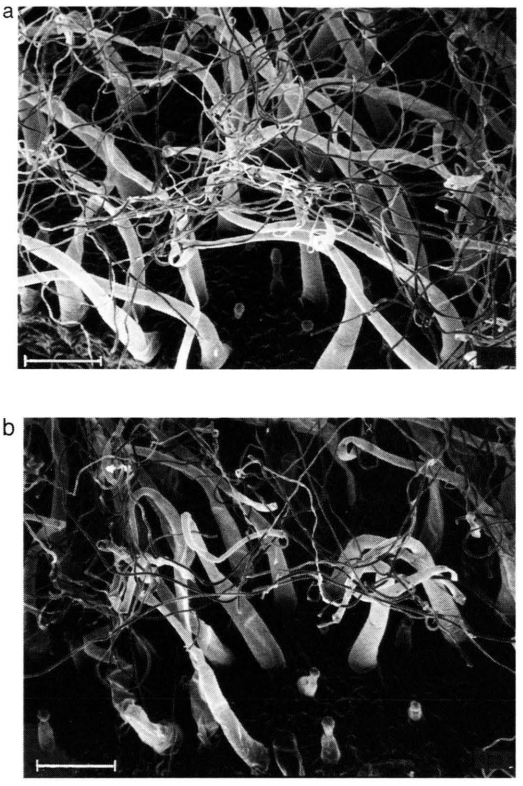


Fig. 2A. SEM pictures of control and treated leaf epidermises after 15 days of UV-B exposure. (a) control leaves. (b) treated leaves. (All 172x; bar = 100 μm).

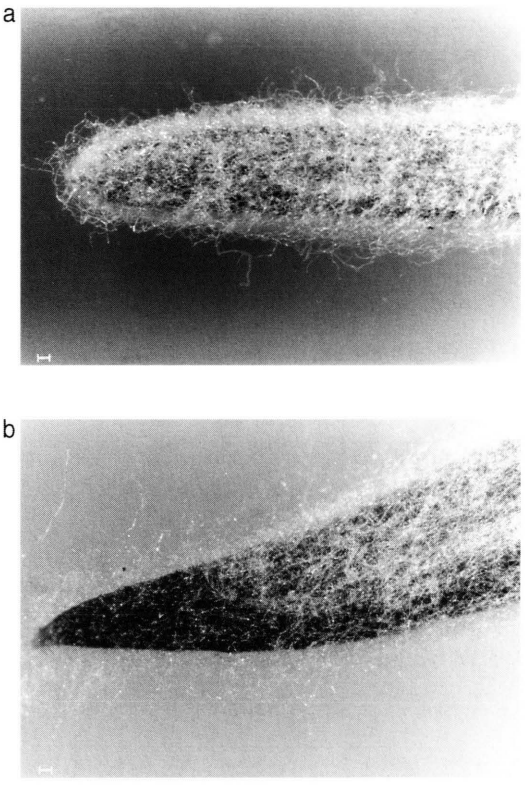


Fig. 2B. Light microscope pictures taken after 15 days of UV-B exposure. (a) control leaves. (b) treated leaves. (All 17x; bar = 100 μm).

changes on the adaxial epidermis of treated leaves (Fig. 2A). It seems that a reduction in epicuticular hairs may be induced by UV-B radiation. This observation was also noted under the light microscope for treated and control leaves (Fig. 2B). Because of the small number of leaves examined it is not possible to elaborate further conclusions but similar findings about the damaging effects of UV-B radiation on epidermal structures have been reported in *Raphanus sativus* and *Hordeum vulgare* (Tevini *et al.*, 1981). UV-B radiation has also been thought to cause partial collapse of the adaxial epidermis by damaging internal structures (Cen and Bornman, 1990). Another physiologically important process such as plant growth appeared to be affected. Many UV-B effects on plants have been described in the literature as damaging or negative effects. In contrast, in this study a clear positive effect on plant growth expressed as stem elongation was noticed. This was the most remarkable effect observed in UV-B irradiated plants. After 15 days of treatment a statistically significant increase in stem height can be observed in the treated plants (Fig. 3A). A comparison in the weekly growth rate (Fig. 3B) also indicates a positive effect of UV-B radiation on growth. A linear fluence-dependent response between stem increase and time of exposure is also observed. This positive effect in plant growth is qualitatively similar to that observed in plant shade responses and might be considered as a plant adaptation to UV-B stress. A suite of experimental responses after UV-B irradiation qualitatively related to shade adaptation is found in Middleton and Teramura (1994). Growth promotion by UV radiation has also been found in tomato (*Lycopersicon esculentum*) and radish (*Raphanus sativus*). Tezuka *et al.* (1993) suggested that UV irradiation not exceeding that found in solar UV spectrum may contribute to the promotion of plant growth. Inhibitor effects on growth would be observed when UV dose exceeds the solar UV intensity. They also re-

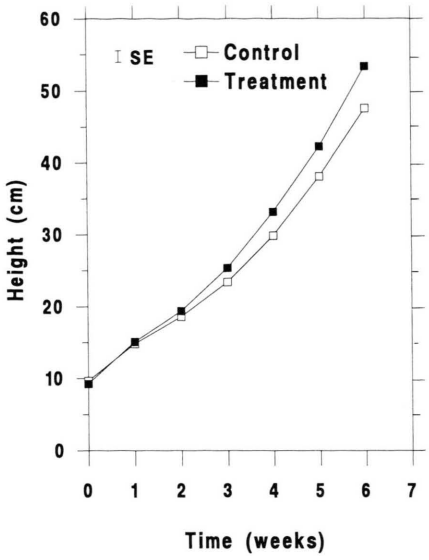


Fig. 3A. Comparison of stem elongation between control and treated plants. Each point represents the average of 70 plants. Means are significantly different at $P < 0.05$ level by LSD in ANOVA. df = 1, F = 72.78, $P < 0.0001$.

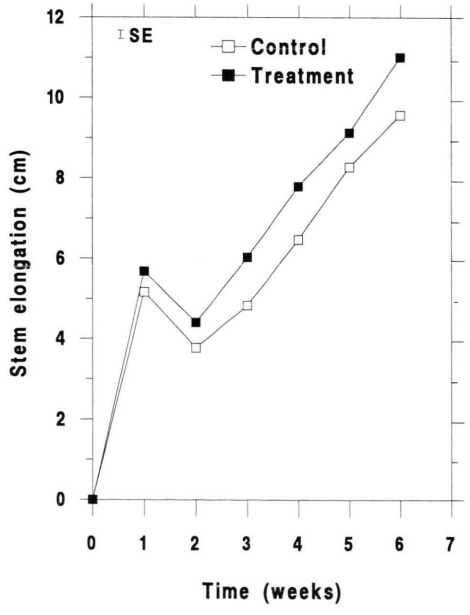


Fig. 3B. Weekly increase in stem elongation (growth rate) for control and treated plants. A linear dependence between increase and UV-B dose is suggested. Each point represents the average of 70 plants. Means are significantly different at $P < 0.05$ level by LSD in ANOVA. df = 1, F = 119.26, $P < 0.0001$.

Table III. Effects of UV-B radiation on photosynthetic pigment content[a].

Pigment[b]	Control	Treatment	F[c]	P[d]
Chlorophyll *a*	2.313±0.176	3.171±0.176[a]	11.87	0.0029
Chlorophyll *b*	1.014±0.063	1.264±0.063[b]	8.01	0.0111
Carotenoid	0.390±0.042	0.514±0.042[c]	4.49	0.0483

[a]Mean concentration (μg pigment/mg fresh leaf; N=10) and standard error of plant pigments after 15 days of UV-B exposure. Means followed by different letters are significantly different at $P < 0.05$ level, as determined by LSD in ANOVA.
[b]Degrees of Freedom=1; F and P (determined in ANOVA) are given for each plant pigment by treatment.
[c]F-value was obtained by dividing the mean square for group by the mean square for residual.
[d]Significance level was determined using 1 and 18 DF.

ported a different effect depending on plant growth stage. In the early growth stage, plants seem to be more sensitive to UV radiation than in the later growth stage. This may be attributed to changes in the chemical composition of plants. Similar effects have also been reported by Seibert *et al.* (1975). Leaf area and leaf fresh weight were also analyzed in *Gnaphalium*. The physiological development of new leaves seems to be affected by UV-B radiation as evidenced in lower values observed in the treated plants. Similar effects were observed in leaf biomass accumulation, but the data obtained were not statistically significant.

The effect of UV-B radiation on the light harvesting complexes has often been reported. However, these studies show inconsistent results on the relative change in the constituent photosynthetic pigments. In this study, the photosynthetic pigment content seems to be altered after UV-B irradiation. Table III shows the effects on pigment content after 15 days of treatment. There is an increase in pigment content in Chl a, Chl b and carotenoid. Carotenoid content has been correlated with UV-B absorbing compounds, photosynthesis and productivity (Middleton and Teramura, 1994). These effects on light harvesting complexes have been attributed to UV-B irradiation and may be considered as a plant strategy to dissipate this harmful radiation from inner tissues, avoiding irreparable damage to photosynthetically important membrane systems (Murphy, 1983; Melis *et al.*, 1992).

Conclusion

The results of this study support the hypothesis of the role of epidermal flavonoids in plant responses to environmental stress. Carotenoids may also be involved in plant defense as well as influencing other plant physiological processes. In this way, plants seem to be using a combination of responses as a full strategy in order to avoid the action of this damaging radiation.

Acknowledgments

We thank the Chilean government for a Ph.D. grant to one of us (P.C.) and to the Universidad de Magallanes for financial support given.

Adamse P. and Britz S. (1992), Spectral quality of two fluorescent UV sources during long-term use. Photochem. Photobiol. **56**, 641–64.

Ali E., Bagchi D. and Pakrashi S. C. (1979), New flavonoids from *Anaphalis araneosa*. Phytochemistry **18**, 356–357.

Beggs C. L., Stolzer-Jehle A. and Wellman E. (1985), Isoflavonoid formation as an indicator of UV stress in bean (*Phaseolus vulgaris* L.) leaves. Plant Physiol. **79**, 630–634.

Bohlmann F. and Ziesche J. (1980), Neue Diterpene aus *Gnaphalium-arten*. Phytochemistry **19**, 71–74.

Campos J. L., Figueras X., Piñol M. T., Boronat A. and Tiburcio A. F. (1991), Carotenoid and conjugated polyamine levels as indicators of ultraviolet-C induced stress in *Arabidopsis thaliana*. Photochem. Photobiol. **53**, 689–693.

Cen Y-P. and Bornman J. F. (1990), The response of bean plants to UV-B radiation under different irradiances of background visible light. J. Exp. Bot. **41**, 1489–1495.

Cen Y-P., Weissenböck G. and Bornman J.F. (1993), The effects of UV-B radiation on phenolic compounds and photosynthesis in leaves of *Brassica napus*. In: Physical, Biochemical and Physiological Effects of Ultraviolet Radiation on *Brassica napus* and *Phaseolus vulgaris* . Thesis, University of Lund, Lund, Sweden. Section IV, 1–14.

Chapelle E. W., Kim M. S. and Mc Murtrey III J. E. (1992), Ratio analysis of reflectance spectra (RARS): an algorithm for the remote estimation of the concentrations of chlorophyll *a*, chlorophyll *b*, and carotenoids in soybean leaves. Remote Sens. Environ. **39**, 239–247.

Coohill T. P. (1989), Ultraviolet action spectra (280 to 380 nm) and solar effectiveness spectra for higher plants. Photochem. Photobiol. **50**, 451–457.

Cuadra P., Fajardo V., Urzúa A., Muñóz O. and Arrieta A. (1994), Determination of the effect of 8-*O*-(2-methyl-2-butenoyl)-5,7-dihydroxy-3-methoxyflavone from *Gnaphalium robustum* on growth of *Escherichia coli* K-12 by optical density and electrical conductance measurements. Planta Med. **60**, 598–599.

Cuadra P. (1989), Estudio Químico del Exudado de *Gnaphalium robustum* Phil. y Determinación de Algunas de sus Propiedades Biológicas. pp. 130. M.Sc Thesis, Facultad de Ciencias, Universidad de Santiago, Santiago, Chile.

Demming-Adams B. (1990), Carotenoids and photoprotection in plants: a role for the xanthophyll zeaxanthin. Biochim. Biophys. Acta **1020**, 1–24.

Giuffra E., García-Madrid R. and Latorre de la Cruz I. (1987), Native plants in Chilean folk-medicine. Ann. R. Acad. Farm. **53**, 296–301.

Hänsel R., Rimpler H. and Schwarz R. (1967), Zur Frage des *Helicrysum*-auronols eine Berichtigung. Tetrahedron Lett. **8**, 735–738.

Harborne J. B. (1991), The chemical basis of plant defense. In: Plant Defenses against Mammalian Herbivory. (R. T. Palo, and C. T. Robbins ed.). CRC Press, Boca Raton, USA, pp. 45–59.

Harborne J. B. (1993), Introduction to Ecological Biochemistry, 4th ed. Academic Press, London.

Hasan M. C., Healey T. M. and Waterman P. G. (1982), Kolavane and kaurene diterpenes from the stem bark of *Xylopia aethiopica*. Phytochemistry **21**, 1365–1368.

Hedin P. A. and Waage S. K. (1986), Role of flavonoids in plant resistance to insects. In: Plant Flavonoids in Biology and Medicine; Biochemical, Pharmacological, and Structure- Activity. (R. A. Liss, Inc., (ed.). Academic Press, New York, pp. 87–100.

Hiscox J. D. and Isrealtam G. F. (1979), A method for the extraction of chlorophyll from leaf tissue without maceration. Can. J. Bot. **57**, 1332–1334 .

Hoffmann A. (1978), Flora silvestre de Chile. Zona Central. Fundación Claudio Gay, Santiago, Chile.

Hoffmann J. J., Kingsolver B. E., Mc Laughlin S. P. and Timmermann B. N. (1983), Recent advances in phytochemistry. In: Phytochemical Adaptations to Stress. (B. N. Timmermann, C. Steelnik, and F. A. Loewus eds.). Plenum Press, New York, pp. 251–271.

Karban R. and Myers J. H. (1989), Induced plant responses to herbivory. Annu. Rev. Ecol. Syst. **20**, 331–348.

Knogge W. and Weissenböck G. (1986), Tissue-distribution of secondary phenolic biosynthesis in developing primary leaves of *Avena sativa* L. Planta **167**, 196–205.

Krinsky N. I. (1979), Carotenoid protection against oxidation. Pure Appl. Chem. **51**, 649–660.

Mabry T. J., Markham K. R. and Thomas M. B. (1970), The Systematic Identification of Flavonoids. Springer-Verlag, Berlin.

Markham K. R. (1982), Techniques of Flavonoid Identification. Academic Press, London, p. 65.

Melis A., Nemson J. A. and Harrison M. A. (1992), Damage to functional components and partial degradation of photosystem II reaction center proteins upon chloroplast exposure to ultraviolet-B radiation. Biochim. Biophys. Acta. **1100**, 312 320.

Middleton E. H. and Teramura A. H. (1994), Understanding photosynthetisis, pigment and growth responses induced by UV-B and UV-A irradiances. Photochem. and Photobiol. **60**, 38–45.

Middleton E. M. and Teramura A. M. (1993), The role of flavonol glycosides and carotenoids in protecting soybean from ultraviolet-B damage. Plant Physiol. **103**, 741–752.

Miles D. (1993), Ultraviolet-B radiation effects on leaf flourescence characteristics in cultivars of soybean in photosynthetic responses to the environment. In: Photosynthetic Responses to the Environment. Current Topics in Plant Physiology. (H. Y. Yamamoto, and C. M. Smith, eds.). American Society of Plant Physiologists, USA, pp. 136–149.

Murphy T. M. (1983), Membranes as target of ultraviolet radiation. Physiol. Plant. **58**, 381–388.

Neale P. J., Cullen J. J., Lesser M. P. and Melis A. (1993), Physiological bases for detecting and predicting photoinhibition of aquatic photosynthesis by PAR and UV radiation. In: Photosynthetic Responses to the Environment. Current Topics in Plant Physiology. (H. Y. Yamamoto, and C. M. Smith, eds.). American Plant Physiologists, USA, pp. 61–77.

Rau W., Seigner L. and Schrott. (1991), The role of carotenoids in photoprotection against harmful effects of UV-B radiation. Biol. Chem. Hoppe-Seyler. **372**, 539.

Reiche C. (1905), Estudios críticos sobre la flora de Chile. IV. Imprenta Cervantes, Santiago, Chile, p. 62.

Reuber S., Leitsch J., Krause G. H. and Weissenböck G. (1993), Metabolic reduction of phenylpropanoid compounds in primary leaves of rye (*Secale cereale* L.) leads to increased UV-B sensitivity of photosynthesis. Z. Naturforsch. **48C**, 749–756.

Robberecht R. and Caldwell M. M. (1986), Leaf optical properties of *Rumex patientia* L. and *Rumex obtusifolius* L. in regard to a protective mechanism against solar UV-B radiation injury. In: Stratospheric Ozone Reduction, Solar Ultraviolet Radiation and Life. W. C. Worrest, and M. M. Caldwell (eds.). NATO ASI series G: Ecology Sciences. Springer-Verlag, Berlin, pp. 251–259.

San Martín J. (1982), Medicinal plants in central Chile. Economic Botany **37**, 216–227.

Seibert M., Wetherbee P.J. and Job D.D. (1975), The effects of light intensity and spectral quality on growth and shoot initiation in tobacco calus. Plant Physiol. **56**, 130–139.

Schnabl H., Wiessenböck G. and Scharf H. (1986), *In vivo* microspectrophotometric characterization of flavonol glycosides in *Vicia faba* guard and epidermal cells. J. Exp. Bot. **174**, 61–72.

Sisson W. B. and Caldwell M. M. (1975), Lamp/filter systems for simulation of solar UV irradiance under reduced atmospheric ozone. Photochem. and Photobiol. **21**, 453–456.

Tevini M., Braun J. and Fieser G. (1991), The protective function of the epidermal layer of rye seedlings against ultraviolet-B radiation. Photochem. and Photobiol. **53**, 329–333.

Tevini M., Iwanzik W. and Thoma U. (1981), Some effects of enhanced UV-B radiation on the growth and composition of plants. Planta **153**, 388–394.

Tezuka T., Hotta T. and Watanabe I. (1993), Growth promotion of tomato and radish plants by solar UV radiation reaching the earth's surface. J. Photochem. Photobiol. B: Biol. **19**, 61–66.

Tomás-Lorente F., Iniesta-San Martín E., Tomás-Barberán F. A., Trowitzsch-Kienast W. and Wray V. (1989), Antifungal phloroglucinol derivatives and lipophilic flavonoids from *Helichrysum decumbes*. Phytochemistry **28**, 1613–1615.

Torrenegra R. D., Ayda R. A., Pedrozo J. and Fuentes O. (1989), Flavonoids from *Gnaphalium gracile* H. B. K. Int. J. Crude Drug Res. **27**, 22–24.

Torrenegra R. D., Pedrozo J., Robles J., Waibel R. and Achenbach H. (1992), Diterpenes from *Gnaphalium pellitum* and *Gnaphalium graveolans*. Phytochemistry **31**, 2415–2418.

Urzúa A. and Cuadra P. (1989), Flavonoids from resinous exudate of *Gnaphalium robustum*. Bol. Soc. Chil. Quím. **34**, 247–251.

Urzúa A. and Cuadra P. (1990), Acylated flavonoid aglycones from *Gnaphalium robustum*. Phytochemistry **29**, 1342–1343.

Wellman E. (1975), UV dose-dependent induction of enzymes related to flavonoid biosynthesis in cell suspension cultures of parsley. FEBS Lett. **51**, 105–107.

Wilhem de Mosbach E. (1992), Botánica Indígena de Chile. Andrés Bello Ed., Santiago, Chile.