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INCREASES IN SURFACE FLAVONOLS AND PHOTOSYNTHETIC PIGMENTS IN *GNAPHALIUM LUTEO-ALBUM* IN RESPONSE TO UV-B RADIATION

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Key Word Index—*Gnaphalium luteo-album*; Compositae; surface flavonoids; photoresponse; UV-B radiation; calycopterin; 3′-methoxycalicopterin; gnaphaliin.

Abstract—Biochemical and physiological changes in *Gnaphalium luteo-album* plants were analysed in response to two different irradiance levels of UV-B. Low irradiance treatments showed increases in stem elongation, in chlorophyll A and B and carotenoid contents, and in the absorbance of vacuolar phenolics. Significant increases were observed in the concentrations of two surface flavonols, calycopterin and 3′-methoxycalycopterin, which were identified for the first time in this species. By contrast, a third surface flavonol, gnaphaliin, decreased in amount. High irradiance treatments inhibited plant growth but still increases in surface flavonols were observed. Our results support the view that surface flavonols are produced by plants as a protective device to shield the leaf from damage by UV-B radiation.

INTRODUCTION

Since the discovery of the spring season 'ozone hole' over Antarctica, concern has developed about the effects of UV radiation on living organisms. Several detrimental effects of UV-B radiation on plants have been recorded. These include biomass reduction [1], decrease in pollen germination and flowering [2], reduction in photosynthetic activity [3], photomorphogenic effects [4] and modification of plant enzyme content [5]. Additionally, alterations to the leaf surface structure [6], variations in epicuticular wax composition [7], increased contents of UV-B absorbing vacuolar pigments [8, 9] and changes in surface flavonoids [10] have been reported. There is also some evidence that UV-B damage is affected by concomitant exposure to high or low photosynthetic active radiation (PAR) irradiance levels [11]. In the same way, morphological/anatomical features of leaves and the previous exposure of plants to intense sun radiation in their natural habitats may play an important role in plant defence against enhanced levels of UV-B radiation.

In previous work, we analysed, for the first time, the effects of UV-B radiation on the leaf surface flavonoids in *Gnaphalium vira-vira*. We chose this Chilean weed because we thought it would be better adapted to high exposure to UV-B radiation, since it grows in a zone under the 'ozone hole'. Leaves from these plants are highly pubescent and, generally, are covered by trichomes or a resinous layer, which is rich in terpenoids and methylated flavones [12]. Flavonoids have often been regarded as being involved in plant defence against UV-B radiation. One of the most important roles might be that of absorbing compounds, screening the solar UV radiation as a natural filter. Because of their location on the leaf surface, as well as in epidermal vacuoles, it seems likely that they are interacting with the environment and, thus, irradiation using UV-B light can modify their content. In order to compare the effects of UV-B radiation in a sun adapted plant, namely *G. vira-vira*, with a plant from less UV irradiated climates, we decided to analyse the responses to UV-B radiation of *G. luteo-album*, a Mediterranean weed which is widely distributed in Central Europe and South Eastern Asia. The results of this study are reported herein.

RESULTS

Structural elucidation of surface flavonols

Two highly methylated flavonols (**2** and **3**) reported for the first time from *Gnaphalium* and a flavonol (**1**)

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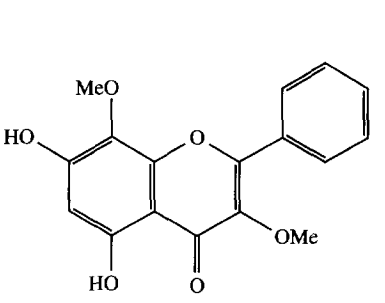

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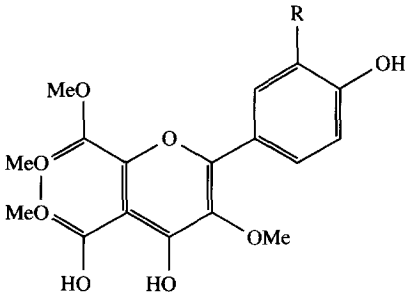

2 R = H
3 R = OMe

common in this genus were isolated from the methylene chloride leaf wash. Identification of these metabolites was made on the basis of NMR, UV and EI mass spectrometry (see Experimental). Thus, **1** is 5,7-dihydroxy-3,8-dimethoxyflavone (gnaphaliin). This flavonoid has previously been found in several *Gnaphalium* species, including the Chilean, *G. robustum* [13]. The spectroscopic data are identical to those reported in the literature [12]. Thus, **2** was identified as 5,4′-dihydroxy-3,6,7,8-tetramethoxyflavone (calycopterin), which has previously been isolated from *Calycopteris floribunda* (Combretaceae) [14] and several other plants [15]. The spectroscopic data are in complete agreement with those reported in the literature [12]. Compound **3** was identified as 5,4′-dihydroxy-3,6,7,8,3′-pentamethoxyflavone (3′-methoxycalycopterin). It has previously been isolated e.g. from *Gutierrezia resinosa* [17] and in *G. microcephala* [15, 16]. The spectroscopic data are identical to those reported in literature.

The three leaf surface flavonols **1–3** now reported in *G. luteo-album* are structurally related to each other, as are the two found earlier in *G. vira-vira* [10]. One of these flavonols, gnaphaliin (**1**) has been reported before in *G. luteo-album*, but the other two flavonols are newly reported in this species. An earlier study of a Turkish specimen of *G. luteo-album* revealed the presence of **1** accompanied by a second lipophilic flavonol, jaceosidin (5,7,4′-trihydroxy-3,6,3′-trimethoxyflavone) [18]; however, we could find no evidence for the occurrence of jaceosidin in our plant material, which originated from Germany.

UV-B effects on surface flavonoids

After 28 days of UV-B irradiation using a low irradiance dose, a simple inspection of plants revealed damage on leaf margins, which were also curlier and had more bronze discolouration than control leaves. The high irradiance treatment increased the bending of leaf margins, caused discolouration of leaves and side-growth. All these effects observed in *G. luteo-album* plants can be attributed to UV-B radiation.

The UV spectrum of the methylene chloride leaf wash showed two main absorption peaks at 234 and 279 nm and a shoulder at 338 nm. TLC of the methylene chloride leaf wash revealed the presence of three compounds with R_fs of 0.65, 0.55 and 0.31. HPLC qualitative analysis using a photodiode array detector confirmed the presence of three main UV-absorbing compounds with R_ts of 10.92, 11.59 and 12.51 min (**1**, **2** and **3**, respectively). In the low irradiance treatment, HPLC quantitation of **1–3** showed that all these metabolites increase as plants grow. Table 1 shows the concentrations of **1–3** in non-irradiated plants. The highest increase is observed for **1**, which is raised to 15 times its original value. The ratio '$R_{2/3}$' also varies with time, from 0.66 to 1.31 at 21 days. In contrast, in irradiated plants (Table 2) this increase was only five-fold; the $R_{2/3}$ changing from 0.89 (initial) to 0.93 (21 days). The concentrations of **2** and **3** are also higher in treated plants than in the controls (Table 2). Statistical analysis confirmed that these differences become significant after 21 days of treatment. At this point, **3** exhibited the maximum value while **2** is still

Table 1. Variation[b] of gnaphaliin, calycopterin and 3′-methoxycalycopterin concentrations[a] in non-irradiated plants

Time (days)	Gnaphaliin[b] **1**	Calycopterin[c] **2**	3′-Methoxycalycopterin[d] **3**
7	22.16[a]	83.17[a]	125.8[a]
14	51.49	121.84	144.39
21	171.17	220.02	167.40
28	324.19	236.79	208.07

[a] Mean concentration (μg flavonoid/30 μl; N = 5) and standard error of flavonoid concentration at different times.
[b] Means are statistically significant by LSD in ANOVA. DF = 3, F = 419.31, P < 0.0001.
[c] Means are statistically significant by LSD in ANOVA. DF = 3, F = 192.75, P < 0.001.
[d] Means are statistically significant by LSD in ANOVA. DF = 3, F = 33.05, P < 0.0001.

Table 2. Effect of UV-B radiation (low irradiance treatment) on gnaphaliin (**1**)[b], calycopterin (**2**)[c] and 3′-methoxy-calycopterin (**3**)[d] content[a]

Irradiation time (days)	Compound	Control	Treatment
7	**1**	0.66	0.68
	2	2.49	3.91
	3	3.77	4.39
14	**1**	1.54	0.98
	2	3.65	4.04
	3	4.33	4.43
21	**1**	5.14	1.91
	2	6.60	7.45
	3	5.02	7.97
28	**1**	9.73	3.23
	2	7.10	8.26
	3	6.24	7.64

[a] Mean concentration (μg flavonoid/30 μl; N = 4)

[b] Means are statistically significant by LSD in ANOVA. DF = 1, F = 475.45, P < 0.0001.

[c] Means are statistically significant by LSD in ANOVA. DF = 1, F = 70.04, P < 0.0001.

[d] Means are statistically significant by LSD in ANOVA. DF = 1, F = 99.37, P < 0.0001.

increasing at 28 days. The reduced values of **1** in treated plants could be because **1** is being used as an intermediate in the flavonoid biosynthetic pathway, giving **2** and **3** after further oxidation and *O*-methylation. Irradiated plants may use **1** as a substrate for the synthesis of **2** and **3**. This assumption, which would also explain the increase of **2** and **3** in treated plants, was tested by giving a high irradiance dose to *G. luteo-album* plants.

Table 3 shows the concentrations of **2** and **3** in control and treated plants at different times after a high irradiance dose. Compound **1** was not detected by HPLC analysis under the conditions of this experiment, so the hypothesis proposed above could not be tested. This is because of the inhibitory effect on plant growth of the high irradiance treatment as manifested in the average height of plants at the beginning of the experiment (low dose: 11.27 cm; high dose: 4.28 cm). This difference in the physiological status of the plants also explains the extremely low amounts of **2** and **3** detected in plants under the high irradiance treatment. Nevertheless, by measuring the variation of **2** and **3** in control and treated plants, a clear increase in the concentration of **3** was observed in irradiated plants, reaching a maximum after 21 days. Statistical analysis confirmed that differences become significant after 7 days of irradiation (Table 3). On the other hand, the concentration of **2** was lower. This suggests that **2** is being used as substrate in the biosynthesis of **3**. A possible explanation for these findings can be found in the UV absorption spectra of these compounds. Under the low irradiance treatment, plants can use both compounds as UV-B protectors which are efficient enough to dissipate the low dosage. But under a higher dose of UV-B light, the absorption peak maxima of **3** (280 nm), well inside the UV-B range, can dissipate this radiation more efficiently than **2**, whose absorption peak maxima is just outside this range (278 nm). The known bathochromic effect that the methylation process produces in the UV spectra of aromatic compounds [19, 20], contributes to the defence strategy mechanism against UV-B radiation.

Table 3. Effect of UV-B radiation (high irradiance treatment) on calycopterin[b] (**2**) and 3′-methoxycalycopterin[c] (**3**) content[a]

Irradiation time (days)	Compound	Control	Treatment
7	**2**	0.42	0.40
	3	0.42	0.58
14	**2**	0.49	0.41
	3	0.56	0.67
21	**2**	0.51	0.54
	3	0.60	0.80
28	**2**	0.57	0.65
	3	0.53	0.65

[a] Mean concentration (μg flavonoid/30 μl; N = 5) at different times.

[b] Means are statistically significant by LSD in ANOVA. DF = 1, F = 4.33, P < 0.0468.

[c] Means are statistically significant by LSD in ANOVA, DF = 1, F = 25.83, P < 0.0001.

Internal UV-B absorbing phenolics

The UV spectrum of methanol extracts of the internal leaf phenolics showed two major absorption peaks at 303 and 331 nm and three minor peaks at 400, 421 and 445 nm. TLC analysis of methanol extracts showed two compounds with a light blue to green fluorescence under UV/NH_3 and R_f = 0.75 and 0.61, typical of caffeic acid esters. Water-soluble flavonoids were only very minor components. Quantitation of these phenolics was carried out following the procedure of Caldwell [21], and Mirecki and Teramura [22]. Table 4 shows the absorbance at 300 nm of methanol extracts of control and treated plants, after the low irradiance treatment. A clear increase in the absorbance of irradiated plants is observed. Differ-

Table 4. Effect of UV-B radiation (low irradiance treatment) on internal UV-B absorbing phenolics (A_{300})[a,b]

Irradiation time (days)	Control	Treatment
7	0.129	0.140
14	0.109	0.162
21	0.097	0.185
28	0.095	0.171

[a] Mean concentration (AU; N = 10) of internal UV-B absorbing pigments at different times.

[b] Means are statistically significant by LSD in ANOVA. DF = 1, F = 95.35, P < 0.0001.

ences become statistically significant after 7 days of treatment. The maximal effect is observed after 21 days and irradiation over a longer period of time did not increase this response. This is a well known plant response to UV-B radiation, which is based on the absorption bands of hydroxycinnamic acid esters, being inside the UV-B range. Thus, these plants respond to UV-B stress by synthesising greater amounts of vacuolar metabolites which can efficiently dissipate high levels of this radiation. The effect of a high irradiance treatment on the internal phenolic fraction after 21 days was to show an increase from 0.442 ± 0.021 (control) to 0.761 ± 0.021 absorbance units at 300 nm. This increase, also observed in the low irradiance treatment, is in general agreement with the results of similar measurements made on other plant species [8–10].

Physiological effects

The photosynthetic pigments, chlorophyll a, chlorophyll b and carotenoids have increased values in treated plants compared with controls. Table 5 shows the effects of the low irradiance treatment after 21 days of exposure. The highest increase is observed in chlorophyll b (49.65%). There is also a significant increase in chlorophyll a (37.41%) and in carotenoids (32.31%). These results are in agreement with reports about increases in photosynthetic and auxiliary pigments after UV-B radiation [10]. This response may be considered as a plant strategy to protect the photosynthetic machinery from harmful radiation. Through this mechanism, and with a greater amount of light harvesting pigments, plants can dissipate UV-B radiation from the inner tissues.

Besides effects on the photosynthetic and accessory pigments, plant height is affected by UV-B radiation (Fig. 1). The steady growth observed in the control plants is increased in treated plants. However, when a high irradiance dose of UV-B radiation was given to plants, a negative effect was observed in plant height

Table 5. Effects of UV-B radiation (low irradiance treatment) on photosynthetic pigment content[a]

Pigment[b]	Control	Treatment	F[c]	P[d]
Chlorophyll a	71.339 ± 6.420	98.028 ± 6.420^{a}	8.64	0.0217
Chlorophyll b	17.485 ± 1.830	26.166 ± 1.830^{b}	11.25	0.0122
Carotenoid	19.153 ± 1.585	25.340 ± 1.585^{c}	7.62	0.0280

[a] Mean concentration (μg pigment/mg fresh leaf; N = 8) and standard error of plant pigments, extracted with DMSO after 21 days of UV-B exposure. Means followed by different letters are statistically significant 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 8 and 7 DF.

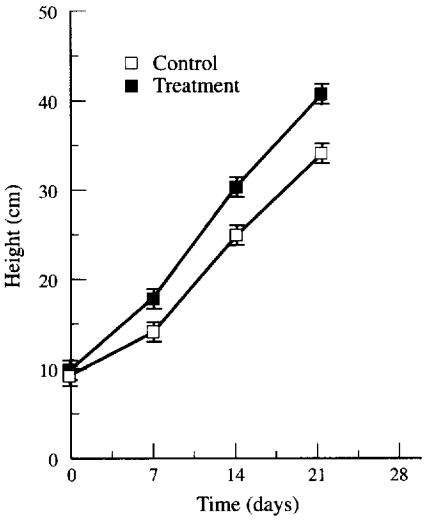

Fig. 1. Comparison of stem elongation between control and treated plants (LI treatment). Each point represents the average of eight plants. Means are statistically significant at $P < 0.005$ level by LSD in ANOVA. DF = 1, F = 24.90, $P < 0.0001$. Variation indicated by bars.

(Fig. 2). Clearly the growth in the treated plants has been negatively affected by this high dose of radiation. This deleterious effect is also observed in a reduction of the fresh weight of leaves. In this way, the negative effects of UV-B radiation on plant biomass are evidenced in two growth variables, i.e. plant height and leaf fresh weight.

These findings support the premise that different doses of UV-B affect the physiology of the plant dissimilarly. Under low levels of UV-B radiation (e.g. as found in natural environments) plants can cope with this amount of radiation and UV-B radiation may contribute to the promotion of plant growth. In

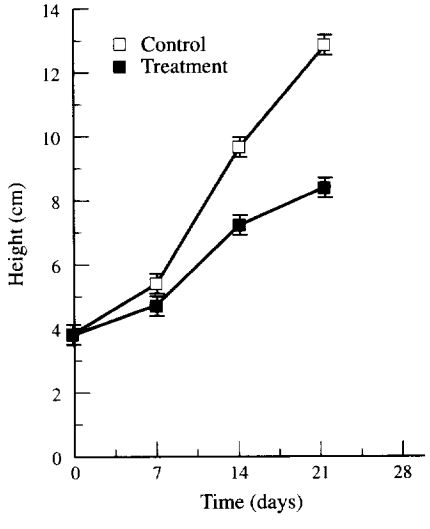

Fig. 2. Comparison of stem elongation between control and treated plants (HI treatment). Each point represents the average of 10 plants. Means are statistically significant at $P < 0.005$ level by LSD in ANOVA. DF = 1, F = 53.61, $P < 0.0001$. Variation indicated by bars.

contrast, when the amount of UV-B radiation reaching the plants exceeds the plant's capacity to dissipate this radiation, detrimental effects are observed. According to the literature, this negative effect on plant growth may be a consequence of changes in the status quo of plant hormones [23] or direct DNA damage [24].

In order to explain the different responses to UV-B radiation of *G. vira-vira* [10] and *G. luteo-album*, we inspected anatomical/morphological differences between leaves of both species. *Gnaphalium vira-vira* plants have leaves which are more than 100% thicker than *G. luteo-album*. This difference can be explained by considering the natural habitat of both species. The former grows in the Southern Hemisphere (Chile) in valleys which are subject to intense sun exposure. By contrast, the latter populates fertile plains or areas near streams in the Northern Hemisphere (Central Europe, Turkey). As reported in the literature, sun adapted leaves have thicker leaf epidermides as an anatomical/morphological barrier against high levels of radiation [22]. Similarly, examination of epidermal surfaces under the light microscope show that leaves of *G. vira-vira* have highly pubescent epidermides and are potentially more protected from external stresses than the less pubescent leaves of *G. luteo-album*.

DISCUSSION

Previous analyses of flavonoids and associated phenolics in plants subjected to UV-B stress have either been carried out on total leaf content or else on the internal leaf constituents [e.g. 7–9]. In the present study on *G. luteo-album* and the earlier one of *G. vira-vira* [10], attention has focused on the surface flavonoids, collected by brief leaf washing in methylene dichloride. Our results show that significant increases occur in these surface components after UV-B irradiance; these may be more important than increases in phenolic absorbance of the epidermal cells. Furthermore, they support the view that surface flavonols produced by plants, at least in part, serve as a protective device to shield the leaf from UV-B radiation damage.

An unexpected feature of the surface flavonols in both *Gnaphalium* species studied is the close biosynthetic relationships between the compounds present. It suggests in *G. luteo-album* that extra oxidations in both A- and B-rings and *O*-methylations occur late in biosynthesis, so that gnaphaliin **1** is a precursor of the more highly substituted flavonols **2** and **3**. This contrasts with the more general view that flavonoids are synthesised in a template system along parallel pathways. But it is conceivable that the pathway of biosynthesis for lipophilic flavonoids, presumably located in the cytoplasm of epidermal cells, is not exactly the same as that for vacuolar flavonoid production.

EXPERIMENTAL

Plant material. Seeds of *G. lueto-album* (provided by the Botanic Garden at Marburg, Germany) were sown on several plastic trays (36 × 24 cm) containing a John Innes no. 1 plus extra sand compost mix. After 10–12 days, 7 seedlings were transplanted into individual plastic pots (26 cm^2) filled with the same compost mix. Plants were watered every 1–2 days. Ambient daytime temps inside the UV-B chamber were 17–25° (cold days) and 28–38° (warm days). All these values were under the maximum temps measured inside the greenhouse.

Light sources. Supplemental to greenhouse sunlight ($PAR_{(400-700)}$: 500–1000 $\mu mol\ m^{-1}$), UV radiation was provided by two (low irradiance; LI: 1.60 mW m^{-2}) and four (high irradiance; HI: 4.50 mW m^{-2}) fluorescent tubes (Philips TL 20 W/12; 220 mm × 1000 mm). UV-B supplementation was obtained by shielding these tubes with presolarised (7 hr) cellulose film (0.075 mm) which cuts off all wavelengths below 280 nm. In order to keep the spectral quality of irradiance output relatively constant, these tubes were 70 hr preburnt [25, 26]. The spectral irradiance levels of plant height below the lamps were measured with a spectroradiometer (Optronics 742, Meteorology Department, The University of Reading) between 250–500 nm, at 5 nm intervals. The spectroradiometer was calibrated using a NIST traceable 1000 W tungsten filament quartz halogen lamp.

UV-B treatments. Plants were distributed in two groups inside the experimental chamber. They were exposed daily to 9 hr of UV-B irradiation (around 12.00 noon) and harvested at different times as analysis required. Lamps were placed on a mobile rack and hung 30 cm above the plants. For spectral quality of the fluence rate, see ref. [10]. Pot positions were randomised within each group every 2 days to minimise position effects. Once a week, lamp rack height was adjusted to maintain the UV irradiance levels. After irradiation, UV-absorbing films were removed in order to give the same light condition to all plants.

Extraction of surface flavonoids. Whole plants (aerial parts) were extracted individually by dipping them in cold CH_2Cl_2 (*ca* 300 ml) for 10–15 sec. Extracts were filtered and then concd to dryness in a rotevaporator at 30°. Residues were kept at 5° for further chromatographic analysis.

Extraction of internal phenolics. Leaves were extracted by grinding them with a pestle and mortar with 2 ml of MeOH–H_2O–HCl (79:20:1). Homogenates, combined with a further washing of the pestle and mortar with 1 ml of the same solvent were centrifuged at 3000 rpm for 10 min. Supernatants were then filtered and evapd to dryness at 40°. Residues were redissolved in MeOH and *A* at 300 nm measured.

Extraction of photosynthetic pigments. Several leaf samples from different stem positions were analysed. Total chlorophylls and carotenoids were extracted from individual leaves with 5 ml of DMSO for 12 hr

at 65° in the dark [27]. A was determined at 664, 648 and 470 nm on 1 ml of samples and the A spectrum recorded between 250–700 nm. Photosynthetic pigment concentrations were calculated according to equations given in ref. [28].

Chromatographic analysis. CH_2Cl_2 leaf wash (0.216 g) was pre-adsorbed with 5 g of Kieselgel 60 and then loaded onto a glass column packed with 30 g of the same adsorbent. The column was developed with petrol (40–60°)-EtOAc (9:1); 47 fr (25 ml) were collected and monitored by TLC using petrol–EtOAc (3:1). Fr 13–16, 17–23, 24–26 and 27–33 were further sepd and purified by prep. TLC using $CHCl_3$–MeOH (199:1) and $CHCl_3$–MeOH (197:3), respectively. After final purification by Sephadex CC, recrystallisation from MeOH yielded 0.68 mg of **1**, 0.82 mg of **2** and 0.77 mg of **3**.

5,7-*Dihydroxy*-3,8-*dimethoxyflavone* (*gnaphaliin*) (**1**). ^{1}H NMR: δ 8.11 (*m*, 2H, H-2′, H-6′), 7.22 (*m*, 3H, H-3′, H-4′, H-5′), 6.45 (*s*, 1H), 4.02 (*s*, 3H,8-OMe), 3.91 (*s*, 3H,3-OMe). EIMS *m/z* (rel.int.): 314.39 ([M]$^+$83; $C_{17}H_{14}O_6$), 299 ([M−15]$^+$100), 271 ([M−43]$^+$12), 256 (8), 183 (A_1^+ 8), 172 (11), 157 (7), 149 (23), 139 (15), 129 (10), 115 (11), 111 (12), 105 (B_2^+, 18), 77 (11). UV λ_{max}: 275,356. [+$AlCl_3$]: 285,335,442. [+$AlCl_3$+HCl]: 283,332,420. [+NaOAc]: 283,384. [+NaOAc+H_3BO_3]: 275,356.

5,4′-*Dihydroxy*-3,6,7,8-*tetramethoxyflavone* (*calycopterin*) (**2**). This was crystallised as yellow needles (mp. 227–228°). ^{1}H NMR: δ ppm: 12.4 (*s*, 1H,D_2O exchangeable, 5-OH); 8.12 (*d*, 2H, J = 7.0 Hz, H-2′, H-6′); 6.99 (*d*, 2H, J = 7.0 Hz, H-3′, H-5′); 4.12 (*s*, 3H, OMe); 3.96(2) (*s*, 3H,OMe); 3.96(0) (*s*, 3H,OMe); 3.89 (*s*, 3H,OMe). ^{13}C NMR: HMBC (heteronuclear long correlation) and NOESY. EIMS *m/z* (rel.int.) = 374.41 (M^+: 72%; $C_{19}H_{18}O_8$ requires 374); 359 (M^+−15: 100); 329 (M^+−45: 7); 301 (5); 211 (A_1^+: 8); 183 (A_2^+: 6); 149 (B_1^+: 5); 121 (B_2^+: 13). UV λ_{max}: 278,340. [+$AlCl_3$]: 281,311,361,417. [+$AlCl_3$+HCl]: 281,311,361,417. [+NaOAc]: 277,343. [+NaOAc+H_3BO_3]: 278,341. [+KOH]: 278,357,407.

5,4′-*Dihydroxy*-3,6,7,8,3′-*pentamethoxyflavone* (3′-O-*methoxycalycopterin*) (**3**). This was crystallised as yellow needles (mp 168–169°). ^{1}H NMR: δ ppm: 13.12 (*s*, 1H,D_2O exchangeable,5-OH); 8.07 (*dd*, 1H,J = 2.0 and 8.0 Hz, H-6′); 8.01 (*d*, 1H, J = 8.0 Hz, H-2′); 7.39 (*d*, 1H, J = 8.0 Hz, H-5′); 4.12 (*s*, 3H, 7-OMe); 4.06 (*s*, 3H,OMe); 4.05 (*s*, 3H,OMe); 4.04 (*s*, 3H,OMe); 3.92 (*s*, 3H,3-OMe). ^{13}C NMR: NOESY. EIMS *m/z* (rel.int): 404.48 (M^+:74.0%; $C_{20}H_{20}O_9$ requires 404); 389 (M^+−15: 100); 359 (M^+−45: 5); 331 (5); 227 (A_1^++1: 1); 211 (A_1^+−Me: 9); 183 (A_2^++1: 7); 151 (B_2^+: 12); 149 (B_1^++1: 9); 135 (6); 97 (59); 84 (5); 83 (8). UV λ_{max}: 262, 280, 352. [+$AlCl_3$]: 285, 368, 435. [+$AlCl_3$+HCl]: 285, 368, 435 [+NaOAc]: 272, 425. [+NaOAc+H_3BO_3]: 262, 280, 355. [+KOH]: 270, 396.

HPLC. Qualitative analysis was performed using a programmable photodiode array detector and a C-18 phenyl reverse phase column. Aliquots of 20 μl were injected and the column developed with a gradient of 25 min from 40% A (2% HOAc) to 60% B (MeOH–HOAc–H_2O, 18:1:1). Quantitation of **1–3** chosen as UV markers was performed on MeOH solns of leaf washes, using a programmable multiwavelength detector and a Spherisorb 55 OD52-6544 reverse-phase column. The column was developed by gradient elution within 30 min from 30% A to 60% B. Concentrations of standards in samples were determined by electronic integration. Concentrations of standards were 3.84 μg 30 μl^{-1} for the LI treatment and 22.2 μg 30 μl^{-1} (**1**), 21.6 μg 30 μl^{-1} (**2**) and 21.43 μg 30 μl^{-1} (**3**) for the HI treatment.

Growth variables. Stem elongation was measured from soil level to the shortest leaf of the first stage. Leaf areas were measured using a leaf-area meter (Delta-T Devices Ltd). Leaves used in area and fr. wt measurements were collected from the top of plants (first and second stages). Each harvest was carried out on three different plants; all measurements were performed weekly. Effects of UV-B radiation on plant biomass, expressed as plant fr. wt, was also analysed.

Spectroscopic analysis. NMR were recorded with a Bruker spectrometer at 400 MHz (Phytochemistry Research Laboratories, University of Strathclyde); COSY, NOESY and HMBC correlation expts were also performed with this spectrometer. Spectra for **1** and **2** were run in $CDCl_3$; pyridine (d_6) was used for **3**. EIMS were measured at 90 eV (by J. Eagles, AFRC Institute of Food Research, Norwich).

Statistical analysis. Data were analysed using procedures for a randomised complete block design (rcbd; [29]). Statistical assessment (ANOVA, LSD test) was performed for all measurements using the SAS 6.08 System Statistical Package.

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