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AN INCREASE IN THE LUTEOLIN : APIGENIN RATIO IN *MARCHANTIA POLYMORPHA* ON UV-B ENHANCEMENT

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Key Word Index—*Marchantia polymorpha*, Marchantiaceae; UV-B enhancement; luteolin : apigenin ratio; flavonoids.

Abstract—The effects of varying the UV-B component in ambient light on the liverwort, *Marchantia polymorpha* are reported. Plants grown under conditions of ambient light, ambient light lacking UV-B, and ambient light with a 25% enhancement of incident UV-B showed, with increasing levels of UV-B, a decrease in growth rate, a decrease in the production of gemmae cups and an increase in the proportion of dead thallus. Thallus surviving after three months of summer growth under these conditions showed no statistically significant increase in flavonoid levels with increasing UV-B levels. However, HPLC monitoring of individual flavonoids (all of which are apigenin and luteolin glucuronides) revealed a strong correlation between increasing UV-B levels and an increase in the ratio of luteolin to apigenin glycosides. It is considered unlikely that this change has significantly altered the UV-B screening effectiveness of the flavonoids. Rather, an improved level of antioxidant defence, or a more effective dissipation of absorbed UV energy, are proposed as possible UV-B protectant benefits to the plant. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

In relatively unpolluted atmospheric conditions such as are found in Antarctica and New Zealand, a reduction in stratospheric ozone levels would produce a concomitant increase in incident solar UV-B radiation [1]. Any such increase has the potential to impact negatively on terrestrial plant growth and thus on crop productivity [2–4]. Plants have developed a range of strategies to protect themselves from the deleterious effects of UV-B radiation including light scattering, UV filtration and photo repair etc [3, 4]. Arguably the most significant of these as adjudged from published reports, is that of UV filtration [e.g. 3, 5, 6]. This is usually accomplished by the production of UV absorbing pigments such as flavonoids which are found deposited in the epicuticular waxes as aglycones, or more universally as glycosides in the epidermal cell vacuoles of higher plants. An enhancement of flavonoid levels in response to increased UV-B irradiation has commonly been cited as an effective defence strategy in plants [5, 7]; and indeed, when flavonoids are at low levels or absent, as for example in some *Arabidopsis* and barley mutants [8, 9] accentuated UV-B sensitivity has been observed.

To date, little attention has been paid to the UV-

B protective responses in flavonoid producing lower plants such as liverworts, mosses and ferns, although the fern *Cryptogramma crista* has recently been shown to respond in the field to increased UV-B levels with a rise in flavonoids [10]. Lower plants might be expected to exhibit a more basic and less extensive array of defensive responses to UV-B than do higher plants, and it was with this in mind that we initiated studies on the liverwort, *Marchantia polymorpha* L.

RESULTS AND DISCUSSION

For the present research *M. polymorpha* plants from the same source were on-grown in equivalent sized trays for three summer months. Ambient light was used in which the UV-B (290–320 nm) level was controlled. A previously described [11] UV-irradiation facility, but incorporating three chambers, was used to grow these liverworts. The chambers allowed exposure of the growing plants to: full spectrum ambient light (A) ambient light lacking UV-B (–UVB), and ambient light containing a 25% UV-B enhancement (+UVB). UV-B enhancement was achieved by continuously monitoring levels of solar UV-B in the ambient chamber, and maintaining a constant electronically controlled 25% enhancement over ambient in the +UVB chamber. Mylar sheeting was used to eliminate UV-B from the –UVB chamber

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Plant responses to increasing UV-B levels

After three months of growing under the conditions outlined above, the $-UVB$ liverworts had spread to cover 88% of their tray, whereas the A liverworts covered 72% and the $+UVB$ liverworts only 43%. Dead thallus in the A and especially the $+UVB$ trays indicated that, as the summer progressed and the UV-B intensity increased, some thallus was not able to protect itself sufficiently. The dead tissue as a percentage of tray area was 1.1% ($-UVB$), 2.2% (A) and 13% ($+UVB$). Another difference observed between the liverworts subjected to these three treatments relates to the number of gemmae cups produced. Gemmae cups from which gemmae, or bud-ded plants, emerge, are organs which develop on the upper surface of the thallus. A reduction in the number of gemmae cups per cm^2 reduced from 1.1 ($-UVB$) through 0.9 (A) to 0.64 ($+UVB$). The above responses are all seen as indicators of increasing levels of plant stress resulting from enhanced UV-B levels.

Flavonoid responses to increasing UV-B levels

The flavonoids in *M. polymorpha* thallus have been shown in previous work [12] to comprise a range of flavone glycosides including apigenin 7-mono-*O*- and 7,4'-di-*O*- glucuronides, luteolin 7-mono-*O*-, 4'-mono-*O*-, 7,3'-di-*O*- and 7,4'-di-*O*-glucuronides accompanied by low levels of the two aglycones. These compounds are readily separable by 2D-PC and HPLC. In the current work, individual compounds isolated by 2D-PC were used as standards to identify peaks in the HPLC readout (see Fig. 1). HPLC was then used to quantify individual flavonoids by use of an internal reference and relative response factors. Total flavonoid contents were then obtained by adding the integrated values of all flavonoid peaks. Quantitative flavonoid analyses were performed on each of three samples taken from each chamber on three of four successive bright days in mid-March. Total daily solar UV-B levels were recorded nearby at a regional monitoring station [13] over the period of harvesting and fluctuated by $\pm 25\%$ from day to day due to variations in cloud cover. The averaged results for the nine samples representing each treatment are presented graphically in Fig. 2 (total flavone glycosides). Whilst there appears to be an increase from $-UVB$ to A with a plateau from A to $+UVB$, the variation between samples (as indicated by the standard error bars) is such that this trend is statistically unproven. In Fig. 2 the variation in total apigenin and total luteolin-containing components is also presented. Again there is some statistical uncertainty, although luteolin levels do appear to trend higher with increasing UV-B levels and apigenin levels decrease.

When the ratio of luteolin to apigenin glycosides is calculated, however, a clear and statistically significant ($P < 0.05$) increase is correlated with increasing UV-B levels (Fig 3). This is an unexpected finding

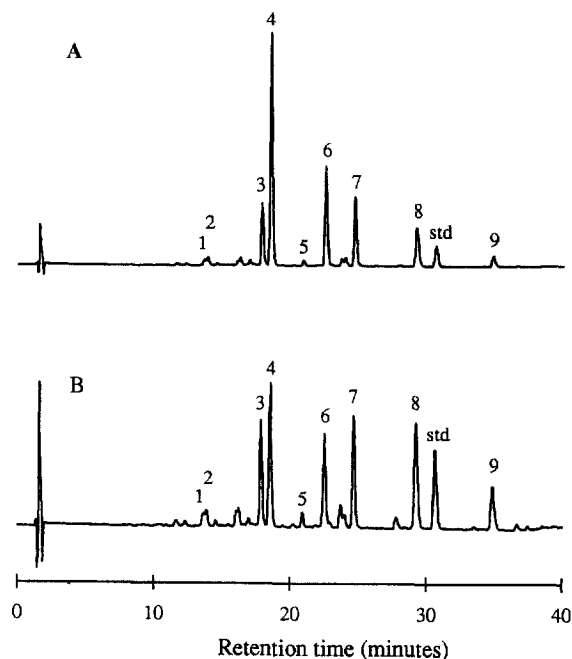


Fig. 1. HPLC display of flavonoid peaks in two extreme examples of *M. polymorpha* grown under (A) ambient light + 25% UV-B and (B) ambient light lacking UV-B. Peak height relativities are valid for comparison only within the same chromatogram since the readouts are not corrected for variables. Peak identifications: 1. Luteolin 7,4'-di-*O*-glucuronide; 2. Apigenin 7,4'-di-*O*-glucuronide; 3. Luteolin 7,3'-di-*O*-glucuronide; 4. Luteolin 7-mono-*O*-glucuronide; 5. Luteolin 4'-mono-*O*-glucuronide; 6. Apigenin 7-mono-*O*-glucuronide; 7. Luteolin 3'-mono-*O*-glucuronide; 8. Luteolin; Std. Naringenin; 9. Apigenin.

as an inspection of the absorption spectra of luteolin and apigenin glycosides [14] indicates that luteolin glycosides are no more effective at absorbing UV-B than are apigenin glycosides. This, taken in con-

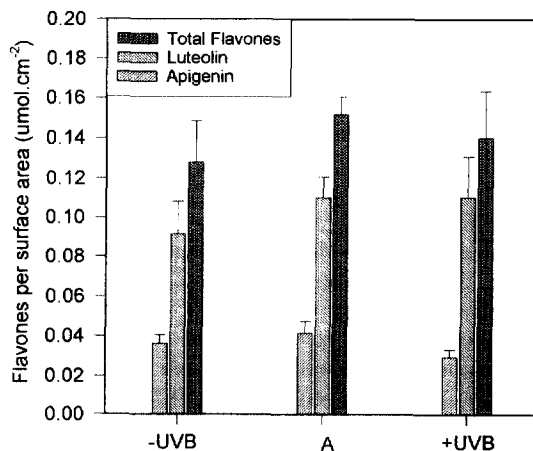


Fig. 2. Variation in the levels of luteolin and apigenin glycosides and total flavone glycosides in *M. polymorpha* thallus with increasing UV-B. Flavonoid molar levels are expressed as apigenin and luteolin 7-glucuronide equivalents per cm^2 .

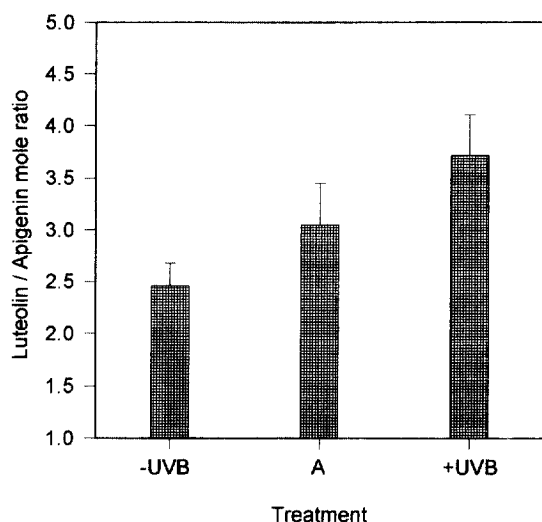


Fig. 3. Variation in the luteolin:apigenin ratio in *M. polymorpha* thallus with increasing UV-B

junction with the conclusion that the overall increase in total flavonoids is statistically uncertain, suggests that the flavonoids in *M. polymorpha* are not responding in the manner expected of a UV-B screen. It is more likely, therefore, that the increased levels of luteolin relative to apigenin serve to protect plant functions in other ways. For example, B-ring orthodihydroxyflavones like luteolin are known to be significantly more effective free radical scavengers/antioxidants than B-ring mono-hydroxyflavones such as apigenin [15, 16] and as such would be more effective in nullifying the potentially deleterious effects of the oxidative free radicals produced in tissue on UV irradiation [17–19]. Alternatively, luteolin may be more effective than apigenin at dissipating absorbed UV energy in a harmless manner, a defence strategy that has previously been proposed [20]. This possibility is currently under investigation.

Although numerous literature reports have appeared in which raised flavonoid levels are considered to be an important plant protection factor against increasing UV-B levels, rarely do these studies involve the monitoring of levels of individual flavonoids [e.g. 6, 21]. Indeed, flavonoid structures are commonly not defined. However, selective enhancement of particular flavonoid types may be more common than accepted to date. To our knowledge, only two reports have appeared in the literature that describe a change in the ratio of the B-ring mono- to di-hydroxyflavonoids [8, 22]. Both detail studies on *Hordeum vulgare* L. (barley) in which it was found that a large increase in the level of a luteolin C-glycoside (luteonarin) was accompanied by a relatively smaller increase in the level of an apigenin C-glycoside (saponarin) on UV-B irradiation in a growth cabinet. In one of these reports, the authors also state that a similar effect, but involving quercetin/kaempferol ratios was observed with *Brassica napus* L. (turnip). In contrast

to the artificial irradiation protocol used by these investigators, the conditions under which the present study was carried out closely simulate the effect of a reducing ozone layer on incident solar UV-B radiation. It is therefore significant that under such "realistic" conditions, a similar influence on flavonoid biosynthesis is observed. Also of significance is the fact that *M. polymorpha*, a bryophyte, is far removed taxonomically and evolutionarily from angiosperms such as *Hordeum* and *Brassica*. As such, the present finding suggests that this response phenomenon may be more widespread than is presently apparent. Indeed, our own as yet unpublished observations on *Oryza sativa* L. (rice) [23] and *Petunia hybrida* [24] indicate persuasively that this is so.

EXPERIMENTAL

Plant material

Marchantia polymorpha thallus material was collected in November 1996 from a natural stand in Silverstream, New Zealand, and was identified as *M. polymorpha* from its distinctive 2D-PC flavonoid profile [12]. Equivalent samples are deposited with Massey University, Palmerston North (MPN 8575,8576).

Plant growth chambers

A plant growth chamber (for specifications, PAR and UV spectral properties etc. see ref. 11) was mounted along the north facing wall of an A-frame laboratory. The chamber was divided into three smaller chambers providing enhanced UV-B, near ambient UV-B (A), and no UV-B (–UVB). The A and +UVB chambers were covered with a clear UV-B transparent teflon film, which admitted natural solar illumination with slightly reduced intensity. The –UVB chamber was clad in mylar to eliminate UV radiation transmission below 320 nm. The +UVB chamber contained 10 Q-Panel UVB-313 40W fluorescent lamps mounted horizontally 0.6 m above the floor. Unenergized lamps were used in the adjacent chambers to provide shading equivalent to that in the +UVB chamber. The radiance of the UV-B tubes was modulated by employing radiometers in both the A and +UVB chambers, to provide a constant ratio of enhancement of UV radiation over ambient throughout the day [11]. The long sides of the chambers faced due north and the lamps were oriented north to south, to minimise shading. A series of fans provided a rapid exchange of air throughout the chambers which did not allow the temperature and humidity to rise above that outside during the day. Small fan heaters maintained a minimum temperature of 8°C overnight to prevent excessive reduction in soil temperature.

Plant growth conditions and measurements

Equal sized clumps (approx. 80 × 80 mm) of thallus were planted into the centre of each of three trays

(350 × 350 mm) of potting mix. One tray was placed in each of the –UVB, A, and +UVB chambers. An automatic system watered plants to excess. At the end of the three month growing period the three trays were photographed, and the total coverage was measured by comparing the weight of a cut-out of the thallus with that of the total tray area. Percentage dead tissue was determined in the same way. Gemmae cups were counted on photographic enlargements using a tally counter.

Data handling

Three samples of *M. polymorpha* thallus were taken from each tray on three of four successive days in mid-March and subjected to quantitative flavonoid analysis as described below. Data from the three samplings were pooled ($n = 9$) and analysed statistically using ANOVA. Statistical significance of trends were assumed when $P < 0.05$.

Flavonoid analytical procedures

The flavonoid profile of *M. polymorpha* was established by 2D-PC on Whatman 3MM paper using *t*-BuOH–HOAc–H₂O (3:1:1) and 15% HOAc as solvents, and NH₃ and NA (diphenyl-boric acid-ethanolamine complex) as detecting reagents. Pure samples of apigenin 7- and 7,4'-glucuronide, and luteolin 7-, 3'-7,3'- and 7,4'-glucuronide were also isolated by this means for use as reference compounds in HPLC. For HPLC analysis, a 28 mm diameter disk of thallus material was cut from each tray with a cork-borer. This was washed with water and the area of the freed up thallus material from each tray was measured from the cut-out of a tracing on paper, followed by weighing and comparison of the weight with that of a standard 2 cm² area. Areas averaged around 10 cm². Plant material was quickly dried at 100°, finely ground, and extracted with 5 ml MeOH–H₂O (7:3) overnight. From each extract was taken 0.5 ml of particle free solution, which was then transferred to an HPLC auto-injection vial containing 25 µl of a standard (1 mg/ml) naringenin solution made up in the same solvent. Analytical HPLC was performed using a Waters 600E solvent delivery system, Waters 996 diode array detector, and a Jasco 851-AS intelligent sampler, results being analyzed using Waters Millennium 2010 software. An injection volume of 20 µl was used with a Merck LiChrospher 100RP-18 endcapped column (5 µm, 4 × 119 mm). Elution (0.8 ml/min, 30°) was performed using a solvent system comprising solvent A (1.5% H₃PO₄ and solvent B (HOAc–CH₃CN–H₃PO₄–H₂O (20:24:1.5:54.5) mixed using a linear gradient starting with 80% A, decreasing to 33% A at 30 min, 10% A at 33 min, and 0% A at 39.3 min. Flavonoids were detected at 352 nm. Under these conditions, reference compounds ex 2D-2PC ran with retention times as follows (mins): Lu-7,4'glur (15.7); Ap-7,4'glur (16.1); Lu-7,3'glur (20.5); Lu-7glur (21.2); Ap-7glur (25.7); Lu-3'glur (28.3); Lu (33.2); Ap (38.7). Response factors (at 352 nm) determined from absorption spectra are: Lu-7glur (1.0); Lu-3'glur and Lu-7,3'glur (1.04); Ap-

7glur (1.14); Ap-7,4'glur (1.4). Integrated areas of individual peaks, after adjustment to account for these response factors and normalisation to an averaged internal standard value, were converted to micrograms using data from a standard luteolin 7-glucuronide solution (1 µg = an integral area of 1,871,665) and then to moles of Ap-7Glur (MW 446) and Lu7-Glur (MW 462).

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