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Long-term exposure to enhanced ultraviolet-B radiation in the sub-arctic does not cause oxidative stress in *Vaccinium myrtillus*

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SUMMARY

The aim of this work was to assess whether or not oxidative stress had developed in a dwarf shrub bilberry (*Vaccinium myrtillus* L.) under long-term exposure to enhanced levels of ultraviolet-B (u.v.-B) radiation. The bilberry plants were exposed to increased u.v.-B representing a 15 % stratospheric ozone depletion for seven full growing seasons (1991–1997) at Abisko, Swedish Lapland (68° N). The oxidative stress was assessed on leaves and stems by analysing ascorbate and glutathione concentrations, and activities of the closely related enzymes ascorbate peroxidase (EC 1.11.1.11) and glutathione reductase (EC 1.6.4.2). The affects of autumnal leaf senescence and stem cold hardening on these variables were also considered. The results showed that the treatment caused scarcely any response in the studied variables, indicating that u.v.-B flux representing a 15 % ozone depletion under clear sky conditions is not sufficient to cause oxidative stress in the bilberry. It is suggested that no strain was evoked since adaptation was possible under such u.v.-B increases. The studied variables did, however, respond significantly to leaf senescence and especially to stem cold hardening.

Key words: Antioxidants, bilberry, cold hardening, u.v.-B radiation, *Vaccinium myrtillus* L.

INTRODUCTION

Thinning of the stratospheric ozone layer increases the flux of ultraviolet-B radiation (u.v.-B; 280–320 nm) reaching the surface of the Earth. The ozone layer over the Northern Hemisphere is decreasing at a rate of 1 % per year (Hofmann & Deshler, 1991). Consequently, a yearly increase in u.v.-B of from 7 % in summer to as much as 35 % in winter was recorded between 1989 and 1993 in Toronto, Canada (Kerr & McElroy, 1993).

As on most living organisms, enhanced u.v.-B radiation is believed to have negative impacts on vegetation. Effects such as altered decomposition rate (Gehrke *et al.*, 1995; Rozema *et al.*, 1997), reduced growth (Johanson *et al.*, 1995*a*), impaired photosynthesis (Bornman, 1989) and damage to cellular macromolecules (Strid, Chow & Anderson, 1994) have been observed. One mechanism behind

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these changes might be oxidative stress arising from exposure to u.v.-B (Bornman & Sundby-Emanuelsson, 1995; Hideg & Vass, 1996; Ormrod, Schmidt & Livingston, 1997).

Most of the experiments on enhanced u.v.-B have been carried out in growth chambers and glasshouses. Responses of plants in controlled conditions, however, may differ from those in the field conditions, due to the presence of visible light and other inconstant environmental factors (Krupa & Kickert, 1989). Northern plants, for example, are expected to be weakly adapted to enhanced u.v.-B radiation because of their natural protection by the lower solar angle and thicker ozone layer compared with their counterparts at lower latitudes (Caldwell, Robberecht & Billings, 1980). A large quantity of the published u.v.-B research focusing on the plant kingdom has been mainly conducted using crops (Stapleton & Walbot, 1994; Liu, Gitz III & McClure, 1995; Takeuchi *et al.*, 1996; Dai *et al.*, 1997) and conifers (Sullivan & Teramura, 1992; Naidu *et al.*, 1993; Petropoulou *et al.*, 1995; Gordon, Percy & Riding, 1998). The aim of this investigation was to assess whether or not u.v.-B under field conditions causes stress in the bilberry (*Vaccinium myrtillus*), one of the main dwarf shrubs in boreal and sub-arctic ecosystems.

The antioxidant defence system of plants provides protection against increased free oxygen radical concentrations (i.e. oxidative stress) in tissues induced by unfavourable environmental conditions such as low temperatures, drought and high light intensities (Bowler, Van Montagu & Inzé, 1992; Allen, 1995). The ascorbate–glutathione cycle removes oxyradicals and their intermediates and is composed of both enzymatic and non-enzymatic mechanisms. The former include ascorbate peroxidase (APOD), dehydroascorbate reductase and glutathione reductase (GR), and the latter include ascorbate (ASA) and glutathione (GSH) (Smith, Vierheller & Thorne, 1989). The antioxidant system might also be one of the defence mechanisms protecting against u.v.-B stress. The ascorbate–glutathione cycle especially is reported to respond to enhanced u.v.-B in algae and herbs (Jansen *et al.*, 1996; Rao, Paliyath & Ormrod, 1996; Dai *et al.*, 1997; Hideg *et al.*, 1997). Therefore, we investigated the hypothesis that u.v.-B would modify the antioxidant metabolism in a shrub, *V. myrtillus*.

Besides responding to various stresses, the antioxidant system undergoes seasonal fluctuation in perennial species. Autumnal leaf senescence in deciduous species (Thompson, Legge & Barber, 1987; Taulavuori & Tolvanen, 1995; Bartoli *et al.*, 1996) and the cold hardening process (Esterbauer & Grill, 1978; Nakagawara & Sagisaka, 1984; Anderson, Chevone & Hess, 1992; Hausladen & Alscher, 1994; Taulavuori *et al.*, 1997a) may alter antioxidant metabolism. Thus this work was conducted during the period of natural leaf senescence and stem cold hardening in order to relate the u.v.-B response of the antioxidant metabolism to its seasonal changes.

MATERIALS AND METHODS

Ultraviolet-B exposure system

To address the impact of u.v.-B radiation on a subarctic heath community, an experiment was established at Abisko, Swedish Lapland (68.35° N, 18.82° E, 360 m above sea level). The dominant species of the experimental site include *Empetrum hermaphroditum*, *Vaccinium myrtillus* L., *Vaccinium uliginosum* and *Vaccinium vitis-idaea* with a sparse cover of *Betula pubescens* ssp. Czerepanovii. A detailed description of the vegetation is given by Sonesson & Lundberg (1974). The experiment was based in open areas of the heath and concentrated on the impact of u.v.-B on the understorey shrub community. Established in the summer of 1990, the experiment has run for seven growing seasons (1991–1997), irradiation starting in the spring a few weeks before all the snow is gone (late April to mid-May) and running until mid-September. Enhanced u.v.-B radiation was supplied evenly from metal frames ($2.5 \times 1.3 \times 1.5$ m high) each with six fluorescent lamps (Q-PANEL UVB-313, Cleveland, OH, USA) as described by Johanson *et al.* (1995b). To derive biologically effective u.v.-B radiation (u.v.-B_{BE}), Caldwell's generalized action spectrum normalized at 300 nm was adopted (Caldwell, 1971). To filter u.v.-C radiation, u.v.-transmitting Plexiglass® (Roehm Gmbh, Darmstadt, Germany) holding a cellulose diacetate filter (0.13 mm, Courtaulds, Derby, UK) was placed beneath each of the lamps. For the control frames window glass was used rather than Plexiglass, which excluded all u.v.-B emission from the lamps. Ultraviolet-B radiation from the lamps was supplied daily, centred around noon and controlled by electronic timers providing stepwise 'square wave' increases. Exposure times were changed every second week to follow the seasonal change in natural u.v.-B radiation. The model developed by Björn & Murphy (1985) and Björn & Teramura (1993) was used to calculate the daily increase in u.v.-B radiation resulting from a 15 % ozone depletion under clear skies at Abisko. The calculated ozone depletion, however, is actually closer to 18–19 % once cloud effects are accounted for.

Harvesting, non-enzymatic and enzymatic antioxidant analysis

There were four replicate plots ($n = 4$) for both the u.v.-B and control treatments, harvesting of which was performed three times: on the 20 July, 10

August and 18 September 1997. Whole bilberry ramets were cut above ground level, frozen in liquid nitrogen and stored in a freezer ($-70\,°C$). Leaves and stems were separated and weighed. Immediately before analysis, samples were ground to a powder with a pestle and mortar in liquid nitrogen.

Total and reduced ascorbate (ASA) were analysed according to Nakagawara & Sagisaka (1984). This assay is based on the reduction of Fe^{3+} to Fe^{2+} by ASA in acidic solution, and a chelate compound formed by Fe^{2+} and bathophenanthroline is detectable at 534 nm. Total ascorbate was determined through a reduction of dehydroascorbate to ascorbate by dithiothreitol. After grinding, 3.75 ml of cold 5 % (w/v) trichloroacetic acid was added to the resulting powder, and grinding was continued until the mixture was completely homogenous. The homogenate was then centrifuged at $16\,000\,\boldsymbol{g}$ ($4\,°C$) for 10 min. Ascorbate concentrations were calculated from standard curves. The proportion of reduced ascorbate (ASA %), which indicates the redox state of ascorbate, was calculated as a proportion of the total ascorbate concentration (ASA/total) × 100.

Concentrations of total and oxidized glutathione were analysed according to Hausladen *et al.* (1990) from samples extracted with 2 % (w/v) metaphosphoric acid, containing 2 mM EDTA. The assay is based on an enzymatic recycling procedure introduced by Griffith (1980). Glutathione concentrations were calculated from standard curves, reduced glutathione (GSH) obtained by subtracting oxidized glutathione (GSSG) from the total concentration. The proportion of reduced glutathione (GSH %), which indicates the redox state of glutathione, was calculated as a proportion of the total glutathione concentration (GSH/total) × 100. Recovery of GSH and GSSG, added to the extraction medium before the extraction of leaf material, was 103 and 97 %, respectively. Stem extraction recovered 96 and 92 %, respectively.

Ascorbate peroxidase (APOD; EC 1.11.1.11) and glutathione reductase (GR; EC 1.6.4.2) activities were determined according to Anderson *et al.* (1992). Proteins were extracted with 5 ml of 50 mM PIPES buffer (pH 6.8), 6 mM L-cysteine hydrochloride, 10 mM D-isoascorbate, 1 mM EDTA, 0.3 % Triton® X-100, 1 % polyvinylpyrrolidine (mol. wt 10000), 1 % polyclar-AT, and 1 drop of antifoam A emulsion (Sigma Biochemicals & Reagents). After centrifugation at $20\,000\,\boldsymbol{g}$ for 15 min ($4\,°C$) the remaining insoluble material was re-extracted with an additional 2 ml of extraction buffer and centrifuged as above. The supernatants of the two extractions were pooled. Immediately before the enzyme measurement, 3 ml of each extract was gel filtered with a Econo-Pac® 10DG column (Bio-Rad Laboratories, Hercules, CA 94547, USA) equilibrated with 50 mM Tris-HCl (pH 7.5), containing 1 mM EDTA and 1 mM D-isoascorbate. The APOD

activity was measured spectrophotometrically by monitoring the ascorbic acid-dependent reduction of H_2O_2 at 265 nm. Assays were performed at $25\,°C$ in a 3 ml reaction mixture containing 1.5 ml of 166 mM HEPES-KOH (pH 7.0), 300 ml 10 mM EDTA, 1.5 mM Na-ascorbate, and 1 mM H_2O_2. The GR activity was measured in a 1 ml reaction mixture at $25\,°C$ as a function of the oxidation of NAD(P)H (170 mM) by GSSG (1 mM) in a Tris HCl-buffer (50 mM, pH 7.5), containing 3.0 mM $MgCl_2$. The reaction was started by adding NAD(P)H and monitored by the decrease in absorbance at 340 nm.

Protein concentration in enzyme extracts was determined according to Bradford (1976).

Data analysis

The effects of treatment and harvest were tested with ANOVA repeated measures (SPSS, 1992).

RESULTS

The results showed that u.v.-B had only a minor (if any) effect on the antioxidant system of the bilberry. No differences in ascorbate concentrations (Fig. 1), ASA % (Table 1), APOD activities (Fig. 2) and glutathione concentrations (Fig. 3) were found between control and u.v.-B treated plants at $P < 0.05$. This holds true for both the leaf and stem data. The proportion of the reduced glutathione (GSH %), however, was lowered by u.v.-B treat-
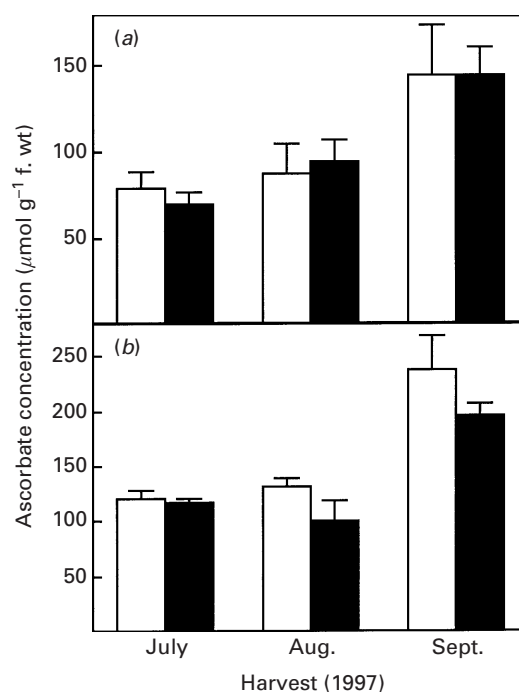

Figure 1. Total ascorbate concentration (μmol g^{-1} f.wt $\pm$ SE, $n = 4$) in bilberry (*Vaccinium myrtillus*) leaves (*a*) and stems (*b*) on three harvest dates (20 July, 10 Aug., 18 Sept. 1997). □, controls; ■, treatment with u.v.-B representing a 15 % ozone depletion.

Table 1. *Proportions of reduced ascorbate (ASA, % ± SE) and reduced glutathione (GSH, % ± SE) in the leaves and stems of bilberry* (Vaccinium myrtillus) *on three harvest dates (20 July, 10 Aug. and 18 Sept. 1997); treatments are control and u.v.-B exposure representative of 15% ozone depletion*

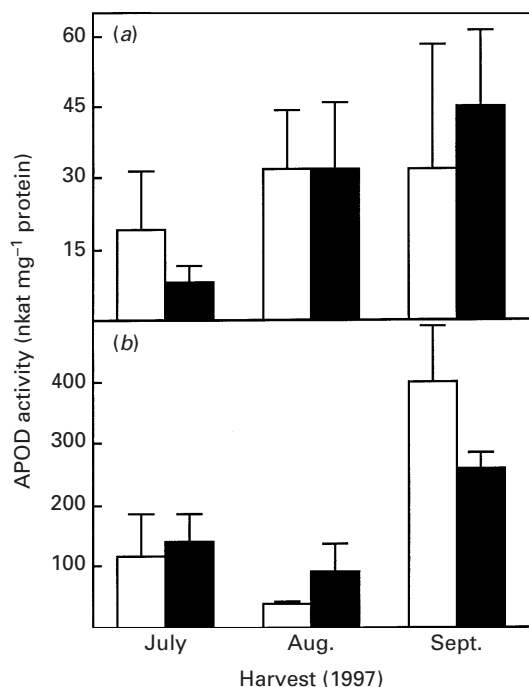|	July		Aug.		Sept.	
	Control	u.v.-B	Control	u.v.-B	Control	u.v.-B
ASA %						
Leaves	70 ± 4	78 ± 8	99 ± 6	93 ± 3	84 ± 6	82 ± 2
Stems	43 ± 2	48 ± 3	59 ± 5	78 ± 7	48 ± 2	50 ± 2
GSH %						
Leaves	83 ± 1	80 ± 1	82 ± 1	85 ± 1	83 ± 1	78 ± 2
Stems	89 ± 1	88 ± 1	92 ± 2	95 ± 1	97 ± 1	94 ± 1

Figure 2. Ascorbate peroxidase (APOD) activity (nkat mg^{-1} protein $\pm$ SE, $n = 4$) in bilberry (*Vaccinium myrtillus*) leaves (*a*) and stems (*b*) on three harvest dates (20 July, 10 Aug., 18 Sept. 1997). $\square$, controls; ■, treatment with u.v.-B representing a 15% ozone depletion.
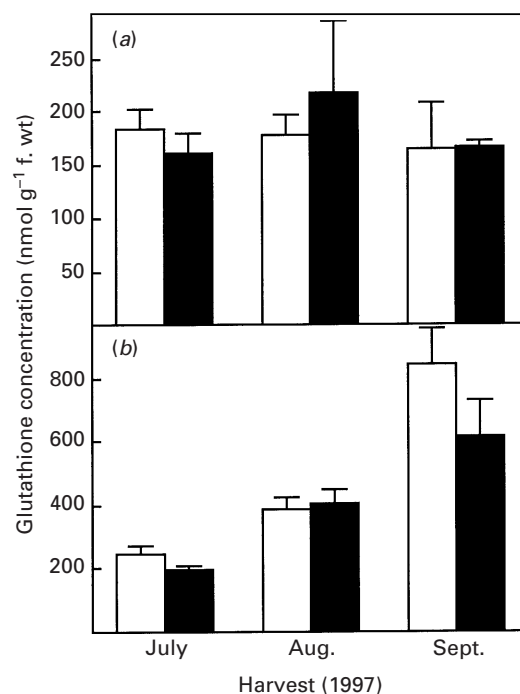
Figure 3. Total glutathione concentration (nmol g^{-1} f.wt $\pm$ SE, $n = 4$) in bilberry (*Vaccinium myrtillus*) leaves (*a*) and stems (*b*) on three harvest dates (20 July, 10 Aug., 18 Sept. 1997). $\square$, controls; ■, treatment with u.v.-B representing 15% ozone depletion.

ment in leaves ($P < 0.05$) and marginally in stems ($P < 0.1$) (Table 1). A marginal reduction ($P < 0.1$) in GR activities was also found in leaves, but not in stems (Fig. 4).

Leaf senescence significantly increased ascorbate concentration ($P < 0.001$) and ASA % ($P < 0.01$) (Fig. 1 and Table 1, respectively). No effect was observed in other variables studied.

Stem cold hardening had a positive impact on all the studied variables. It increased ascorbate concentrations (Fig. 1), ASA % (Table 1), glutathione concentrations (Fig. 3) and GSH % (Table 1) significantly at $P < 0.001$. In addition, APOD (Fig. 2) and GR (Fig. 4) activities increased significantly at $P < 0.01$.

DISCUSSION

Varying responses in antioxidants under u.v.-B exposure have been reported, depending on species and intensity of radiation (Strid, 1993; Rao *et al.*, 1996; Takeuchi *et al.*, 1996; Dai *et al.*, 1997), revealing no uniform response pattern. For example, increased APOD activity was reported in *Arabidopsis thaliana* under enhanced u.v.-B radiation at the level of 18 kJ m^{-1} d^{-1} (Rao *et al.*, 1996). By contrast, u.v.-B exposure at the level of 13 kJ m^{-1} d^{-1} decreased APOD, catalase and superoxide dismutase activities and ascorbate level of *Oryza sativa*, but increased glutathione level (Dai *et al.*, 1997). Enhanced u.v.-B in the current work (max. 2.5–5.8 kJ m^{-1} d^{-1} vs. 2.0–4.6 kJ m^{-1} d^{-1}) had scarcely any effect on the

Figure 4. Glutathione reductase (GR) activity (nkat mg^{-1} protein ±SE, $n = 4$) in bilberry (*Vaccinium myrtillus*) leaves (*a*) and stems (*b*) on three harvest dates (20 July, 10 Aug., 18 Sept. 1997). □, controls; ■, treatment with u.v.-B representing 15 % ozone depletion.
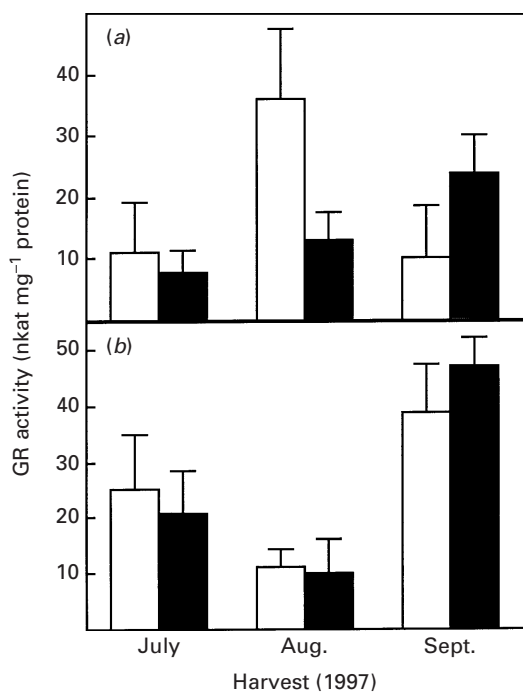
antioxidant defence system (ASA, APOD, GSH, GR) of the bilberry which might suggest that the level of radiation used was not sufficient to induce any overall changes.

The result could also be explained by the adaptability of bilberry to slightly enhanced u.v.-B, especially as the studied plants had experienced it for seven growing seasons. Enhanced u.v.-B radiation representing at 15 % ozone depletion increases u.v.-B absorbing pigments of a subarctic grass *Calamagrostis lapponica* (Gwynn-Jones & Johanson, 1996). Such a pigmentation response might well be the particular adaptation mechanism adequate to cope with a subtle u.v.-B increase, allowing the plant to avoid the oxidative stress condition and consequent response in antioxidant metabolism. Consistent with this view, no antioxidant response was found in this *Calamagrostis* study (unpublished data). In addition, being a perennial species, bilberry may have greater adaptability to environmental stressors compared with annual species: in the context of cold hardening dehydration of cells may pre-adapt plants to u.v.-B stress, since drought stress can ameliorate u.v.-B radiation effects (Tevini, Iwanzik & Teramura, 1983; Teramura, Sullivan & Lydon, 1990). Irrespective of the mechanism, the bilberry is apparently quite tolerant of enhanced u.v.-B radiation representing a 15 % ozone depletion.

Leaf senescence significantly increased ascorbate concentrations and ASA % in this study. Ascorbate is needed in the detoxification of H_2O_2 produced in senescing tissue. It is also a reductant in the regeneration of a-tocopherol and in the zeaxanthin cycle, both of which are part of the protective system in chloroplasts (Alscher, Donahue & Cramer, 1997). The marked increase in total ascorbate concentration and ASA % observed in this investigation thus reflects an enhanced protection against increased level of reactive oxygen species generated during senescence.

All components of the antioxidant system studied generally increased during cold hardening of the stems, indicating enhanced requirements for protection against oxidative stress during this process (Esterbauer & Grill, 1978; Nakagawara & Sagisaka, 1984; Anderson *et al.*, 1992; Hausladen & Alscher, 1994; Polle & Morawe, 1995; Taulavuori *et al.*, 1997*a*; Taulavuori *et al.*, 1997*b*). This result highlights the main outcome of the present investigation; although no response to u.v.-B was obtained, the methods used where shown to be sensitive enough to respond to seasonal changes. According to general stress concepts (e.g. Lichtenthaler, 1996) the response may alternate between positive and negative. In the present work, however, there were no difficulties in interpreting the data, since no particular response to u.v.-B was detected, although several variables and the tissues of two separate organs were studied.

CONCLUSIONS

To our knowledge, this is the first investigation of the antioxidant status of a plant species within a natural community exposed to long-term enhanced u.v.-B radiation conditions. The antioxidant metabolism responded only slightly to u.v.-B exposure, varying responses occurring in GSH % and GR activity, depending on harvest date. In addition, senescence induced varying responses in components of the antioxidant system, and cold hardening almost exclusively induced an increase in the levels of non-enzymatic and enzymatic antioxidants. Thus, it is concluded that the antioxidant system of bilberry is capable of resisting the harmful impact of enhanced u.v.-B radiation at the level imposed in this experiment.

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