Leaf-level responses to ultraviolet-B radiation in *Trifolium repens* populations under defoliation pressure

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ABSTRACT

Pasture plants such as *Trifolium repens* L. (white clover) are exposed to high levels of ultraviolet-B (UV-B) radiation in summer, as well as to frequent defoliation events from grazing animals and pests. This study examined responses in two *T. repens* populations exposed to 16 weeks supplementation of 0 or $13.3 \, \mathrm{kJ \, m^{-2} \, d^{-1}}$ UV-B radiation under controlled environmental conditions. During that period, plants were exposed to two large defoliation events that lasted two and three weeks, respectively. We investigated a number of leaf morphological characteristics, photochemical attributes, as well as aspects of cellular leaf structure. In particular, we sought to explore whether possible differences in these attributes between the two *T. repens* populations could be related to their UV-B responsiveness. Leaf dry mass decreased by 16% in the UV-B-sensitive cultivar 'Huia' under UV-B, whereas the tolerant ecotype 'Tienshan' was unaffected. This differential UV-B response was related to constitutive differences in leaf mass and in leaf area between the two populations. UV-B did not affect specific leaf mass, whereas leaf dry matter content was reduced by 8% in response to UV-B. Measurements of chlorophyll fluorescence revealed no significant effects of UV-B on photochemistry. Results from light microscopy showed that the cellular leaf structure of the *T. repens* populations was not damaged by UV-B. Population-specific structural features included more dome-shaped epidermal cells for 'Tienshan'. We conclude that differential UV-B-responses in *T. repens* populations can occur after defoliation pressure and can be related to differences in leaf characteristics.

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1. Introduction

As a consequence of stratospheric ozone depletion and other anthropogenic and natural factors, increases in clear-sky sunburning ultraviolet-B radiation (UV-B, 290–315 nm) during the last three decades were about 8% at 50°S and 5% at 50°N (McKenzie et al., 2011). UV-B can influence plant processes either through direct damage or via various regulatory effects (Rozema et al., 1999; Potters et al., 2009). The latter includes UV-B-generated morphological responses such as smaller leaf size (Hofmann and Campbell, 2011), decreased leaf mass (Sangtarash et al., 2009), as well as differential UV-B effects on these two attributes, yielding higher

specific leaf mass (Hofmann et al., 2001). There are also indications that leaf dry matter content can be affected by UV-B (Wand, 1995; Laposi et al., 2009). UV-B-induced plant damage includes effects on photochemistry, for example UV-B-generated decreases in photochemical yield ($\Delta F/F_{m'}$) and in intrinsic efficiency of photosystem II (F_v/F_m) (Heijari et al., 2006). This can result from UV-B-induced disruption of chloroplast ultrastructure (Caasi-Lit et al., 1997; Zhu et al., 2002). Other UV-B-induced symptoms of structural damage can include intracellular disintegration and collapsed epidermal cells (Barsig and Malz, 2000).

Only some studies have examined regulatory effects of UV-B on plant cellular and subcellular structure. These effects can include alterations in the number of cell layers and changes in leaf thickening (Kakani et al., 2004; Fagerberg and Bornman, 2005). In studies of the pasture legume *Trifolium repens* L. (white clover) we found that UV-B decreased epidermal cell surface size and that small cell size was linked to UV-B tolerance (Hofmann et al., 2001). Differences in epidermal cell size and shape have been related to differences in light focussing and scattering (Day et al., 1992; Gorton and Vogelmann, 1996; Vogelmann et al., 1996). A survey of 47 plant species showed that *T. repens* epidermal cells had one of the smallest radii of curvature (highly convex outer cell surface), focussing light just below the epidermal layer (Vogelmann et al.,

1996). These studies also suggested that epidermal cell shape can affect the photosynthetic performance of leaves, but little is known about the adaptive and physiological significance of epidermal cell shape for UV-B responsiveness in plants.

In *T. repens* and most other plant species, leaves are the main organs exposed to UV-B radiation. Leaves of pasture plants are also frequently exposed to strong defoliation pressure in summer, when UV-B levels are high and the frequency and severity of defoliation from grazing animals and pests increases, concomitant with decreased plant growth (Andrews et al., 2007). Little is known on the effects of UV-B in plants exposed to defoliation pressure. Defoliation stress can affect various functions in *T. repens*, for instance light interception, stem and root development or carbohydrate accumulation (Hart, 1987; Hofmann et al., 2007). Furthermore, defoliation elicits systemic wound responses in plants and such responses can also be caused by UV-B (Mellway et al., 2009; Sato et al., 2009). It is possible that stress signals from defoliation of mature leaves are transported to the newly developing leaves. It was therefore of interest to determine UV-B effects on newly formed leaves, after a sustained period of UV-B irradiation and defoliation of mature leaves.

Previous studies suggest a fundamental relationship between UV-B responsiveness and competitive strategy in *T. repens* (Hofmann et al., 2000, 2001). The present study aims at further expanding this framework, using intraspecific comparisons to examine possible linkages between UV-B responsiveness and plant specialisation. Intraspecific studies are particularly suited to such investigations as they are not compounded by species-specific differences. The *T. repens* population 'Huia' is a commonly used cultivar bred for agricultural productivity, whilst 'Tienshan' is a slow-growing *T. repens* ecotype adapted to multiple stresses (Hofmann et al., 2000, 2001). Leaf attributes in the two *T. repens* populations were examined at the end of the experimental period, during which the plants had been exposed to 16 weeks of UV-B radiation and two periods of strong grazing pressure. We hypothesised that photochemical, structural or morphological leaf attributes will be related to differential UV-B responsiveness of the two *T. repens* populations under defoliation stress.

2. Materials and methods

2.1. Experimental design

The experimental design was a 2×2 factorial, with two levels of UV-B radiation and two *T. repens* populations. The two UV-B treatments were applied in two large climate-controlled growth chambers which have demonstrated excellent comparability between rooms (Warrington et al., 1999). To further minimise potential room effects, plants and their respective UV-B treatments were rotated several times between the two chambers during the experimental period. To minimise potential location effects underneath the UV rigs, the pots containing the plant material were placed randomly on trolleys which were rotated regularly in the two chambers.

2.2. Plant cultivation and UV irradiation

Stolon cuttings of the *T. repens* populations 'Huia' and 'Tienshan' were planted into separate pots containing 3 kg of soil (Karapoti brown sandy loam), supplemented with 2 g of lime and 10 g of slow-release fertiliser (15-4-10 N-P-K plus micronutrients). Plants were watered daily with an automatic drip irrigation system. Day/night temperatures in the growth rooms were 24 °C and 18 °C, respectively and average relative humidity was 70%. The average photosynthetic photon flux (PPF) during the 14 h daylight period

was 420 μmol m^{-2} s^{-1}, supplied by four 1 kW Sylvania 'metal-arc' high pressure discharge lamps, together with four 1 kW Philips tungsten iodide lamps.

UV-B radiation was supplied by Philips TL 40W/12 RS fluorescent UV tubes, enclosed in cellulose diacetate filters and placed in rigs that were suspended underneath the lighting system. The irradiance levels were 13.3 kJ m^{-2} d^{-1} biologically effective UV-B, normalised to 300 nm (Caldwell, 1971), equivalent to about 25% mid-summer ozone depletion above Palmerston North, New Zealand (40°21′S, 175°37′E). UV radiation was applied from 1 h after onset of the light period until 1 h before darkness. In order to maintain the set UV-B dose, a feedback control system was used to continuously monitor and adjust the output of the UV lamps in response to degradation of the filters and ageing of the tubes (Lindroth et al., 2000). Mylar filter sheets were used to screen out UV-B radiation in the control room, whilst maintaining UV-A levels similar to those in the UV-B treatment room. Plants were subjected to the UV treatments for one month prior to the onset of defoliation. For this, randomly selected mature leaves of each plant were cut with scissors at the base of the petiole. Defoliation was applied in two periods to mimic a rotational grazing regime (Dale et al., 2008). During a defoliation period, all plants were exposed to an increasing level of defoliation pressure, from no leaf removal at the beginning, to the removal of most leaves at the end of the period. The first defoliation period lasted two weeks, followed by a regeneration phase to allow the plants regain their leaf canopy (Oates et al., 2011). The second period of defoliation was applied for three weeks. Compared to the first defoliation period, this additional week of defoliation was due to the larger size of plants in the second period of defoliation. Following this, plants were allowed to recover for one week before the newly formed leaves were harvested for morphological, photochemical and structural examinations.

2.3. Measurements of leaf morphology and photochemistry

Leaf size was measured at the end of the experiment with a leaf area meter (LI-COR Model 3100, Lincoln, Nebraska, USA) on four fully open distal leaf laminae per plant. The leaf laminae were subsequently dried at 80 °C for 48 h for the determination of dry mass. The ratio of lamina dry mass over lamina size was calculated to give specific leaf mass (SLM), and leaf dry matter content was measured as the percentage dry matter (PDM) of the leaf from (lamina dry mass/fresh mass) * 100 (Wand, 1995). These measurements were conducted on ten plants (replicates) for each *T. repens* population under each UV-B treatment. Chlorophyll fluorescence measurements (Hofmann et al., 2001) were performed on the middle leaflets of fully open distal leaves in five replicate samples for each population under each UV-B treatment. Leaves that had been in darkness in the growth chamber for at least 8 h were sampled and immediately stored on moist filter paper in cuvettes sealed with a rubber stopper. Using pulse amplitude modulated (PAM) fluorometry at room temperature (PAM 101 Fluorometer; Walz, Effeltrich, Germany), initial fluorescence in darkness F_o was determined using a measuring light. Following this, maximum chlorophyll fluorescence in dark-adapted leaves F_m and the fluorescence ratio F_v/F_m ($F_v = F_m - F_o$), indicating intrinsic efficiency of photosystem II (PSII) (Maxwell and Johnson, 2000) were determined using a flash intensity of 10 000 μmol m^{-2} s^{-1} (KL1500; Walz). For light-adapted fluorescence measurements, samples were collected in the illuminated growth room and allowed to equilibrate in the cuvette for 4 min at the PPF level equivalent to that in the growth room. Measurements of light-adapted initial (F_t) and maximum ($F_{m'}$) fluorescence were taken to calculate actual efficiency of PSII during illumination (photochemical yield = $\Delta F/F_{m'}$, where $\Delta F = F_{m'} - F_t$) (Maxwell and Johnson, 2000).