Population studies of ultraviolet-B radiation responses in white clover (*Trifolium repens* L.)

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To Angelika and Stephan

Abstract

White clover growing in New Zealand is experiencing increasing levels of ultraviolet-B (UV-B) radiation as a result of ozone depletion. This thesis reports a series of investigations on morphological and physiological responses to UV-B in various white clover populations. In addition, these studies examined UV-B responsiveness in combination with drought and consequences for insect herbivores. Plants were grown in controlled environment rooms with and without supplemental UV-B radiation at a dose of 13.3 kJ m^{-2} d^{-1}, corresponding to a 25% mid-summer ozone depletion above Palmerston North, New Zealand. Morphological measurements included numerous attributes of plant growth and morphogenesis as well as several aspects of leaf structure. Physiological studies investigated both primary and secondary metabolic functions. In general, UV-B reduced components of white clover growth. The white clover populations showed a number of constitutive and UV-B-induced differences in many morphological and physiological attributes. UV-B sensitivity was mitigated by drought and was less pronounced with increasing duration of UV-B exposure. Bioassays revealed differential dietary effects of UV-B-treated foliage on the performance of lepidopteran insects. UV-B effects were also apparent under frequent defoliation. Comparisons of morphological and physiological features showed that white clover UV-B responsiveness was mainly linked to inherent differences in morphology and growth among the populations, while on a physiological level it was more the UV-B-induced differences that conferred UV-B tolerance. In particular, UV-B tolerance of the white clover populations was related to lower constitutive productivity. This was further linked to several constitutive leaf attributes, including small leaf size, small and lens-shaped epidermal cells as well as low leaf water potential. UV-B-induced features linked to UV-B tolerance included high percentage of leaf dry mass, accumulation of UV-absorbing compounds and of total flavonols (particularly quercetin glycosides) as well as increases in leaf water potential. UV-B tolerance was greatest in white clover populations adapted to multiple and severe stresses in the habitat of origin. This series of investigations was used to propose a functional framework, linking UV-B responsiveness to underlying specialisation of the white clover populations.

This thesis has been written with a view to publish much of the presented material. Accordingly, each Chapter represents a self-contained unit, forming the basis of papers published, submitted or prepared for submission.

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Abbreviations

AC	Available carbohydrates
ACR	Average consumption rate
AD	Approximate digestibility
AGR	Average growth rate
$\Delta F/F'_m$	Photochemical yield
DM	Dry mass, dry matter
ECI	Efficiency of conversion of ingested food
F_v/F_m	Intrinsic efficiency of PSII
HCN	Hydrogen cyanide
LAR	Leaf appearance rate
LM	Light microscopy
LWR	Leaf weight ratio
NDF	Neutral detergent fibre
NGP	Number of growing points
NPQ	Non-photochemical quenching
PC	Principal component
PCA	Principal components analysis
PDM	Percent leaf dry mass
PPF	Photosynthetic photon flux
PSII	Photosystem II
q_P	Photochemical quenching
RGR	Relative growth rate
RSR	Root:shoot ratio
SER	Stolon elongation rate
SLM	Specific leaf mass
TEM	Transmission electron microscopy
UV-A	Ultraviolet-A radiation
UV-B	Ultraviolet-B radiation
UV-C	Ultraviolet-C radiation
Ψ_L	Leaf water potential

Chapter 1. Introduction

This Chapter has been published in part in Campbell *et al.* (1999).

The solar UV environment of New Zealand

Solar radiation and ozone

Recently published results from long-term studies provide strong evidence that depletion of the stratospheric ozone layer leads to a net increase in biologically active ultraviolet (UV)-B radiation (290-315 nm) in New Zealand (McKenzie *et al.*, 1999). First reports on the reduction of stratospheric ozone by anthropogenic emissions appeared nearly thirty years ago (Johnston, 1971). The destructive processes in the stratosphere caused by halogen-containing compounds were elucidated by Molina and Rowland (1974), based on earlier studies by Crutzen (1972). This achievement earned those three scientists the 1995 Nobel Prize for chemistry. The catalytic losses of stratospheric ozone in the southern hemisphere ('ozone hole') were first reported a decade later (Farman *et al.*, 1985).

The long and narrow north-to-south orientation of New Zealand covers a relatively wide range of latitude: 34°S to 47°S, equivalent to extending from parts of middle Europe, through southern Europe and to northern Africa. Across this gradient, UV levels differ more between north and south than levels of total solar radiation, the latter ranging around an annual mean daily dose of 14-15 MJ m^{-2} (McKenzie *et al.*, 1996). Most of the country receives about 2000 sunshine hours per year. The peak mean hourly photosynthetic photon flux (PPF) in central New Zealand during summer is approximately 2100 µmol m^{-2} s^{-1}, with a monthly summer average of up to 1700 µmol m^{-2} s^{-1} (Greer and Laing, 1992).

On average, northern New Zealand receives 25% more erythemally-weighted (or 'sunburning') UV than the south and this is mainly due to a smaller solar zenith angle (SZA, determining optical path length) in the north (McKenzie *et al.*, 1996). Regulated by the seasonal changes in the SZA, typical clear-sky daily erythemally-weighted UV

doses range from 0.5 kJ m^{-2} (winter) to 7 kJ m^{-2} (summer) (McKenzie *et al.*, 1996). While the mean annual erythemally-weighted UV dose (ca. 3 kJ m^{-2}) is about half that experienced at the equator, the summer values in the north of New Zealand are similar to equatorial levels (McKenzie *et al.*, 1996). The daily clear-sky mid-summer plant-weighted UV dose above Palmerston North, normalised to 300 nm is approximately 8.4 kJ m^{-2}.

Ozone levels in New Zealand display a maximum in late winter/early spring (September/October, ca. 350-360 Dobson units, DU) and a minimum in late summer/early autumn (February/March, ca. 270 DU) (McKenzie *et al.*, 1996). Thus the maximum ozone levels over New Zealand can be found around the time of greatest depletion over Antarctica, prior to the annual break-up of the polar vortex and spread of this ozone-depleted atmosphere.

Hemisphere comparisons and New Zealand-related factors

Although stratospheric ozone reductions have been measured in both hemispheres, erythemally-weighted UV levels in New Zealand are on average about 40-50% higher compared to similar northern hemisphere latitudes (Table 1-1) (Seckmeyer *et al.*, 1995). This also takes into account differing conditions in cloudiness, aerosol loading and surface albedo. The higher UV levels have been attributed mainly to decreased ozone levels and are seen to result both from lower ozone amounts in the stratosphere over New Zealand as well as from higher levels of tropospheric ozone at the similar northern hemisphere latitudes (Seckmeyer and McKenzie, 1992). Other contributing factors responsible for elevated UV levels in New Zealand compared to similar northern mid-latitudes are seen to be the closer summer Sun-Earth separation and lower aerosol loading of the atmosphere (Seckmeyer and McKenzie, 1992).

Table 1-1. Comparisons of mean daily erythemally-weighted UV-B irradiances (kJ m^{-2} d^{-1} m^{-2}) in summer at similar latitudes in New Zealand and Germany during 1993-1994 (Seckmeyer *et al.*, 1995).

Site	Location	Nov	Dec	Jan	Feb
Lauder, New Zealand	45.0°S, 169.7°E	3.4	4.7	4.8	4.2
		May	Jun	Jul	Aug
Garmisch-Partenkirchen, Germany	47.5°N, 11.1°E	2.5	2.9	3.3	2.7

Cloud cover is one of the most important factors influencing UV levels (Bodeker and McKenzie, 1996). New Zealand is called 'Aotearoa' in the Maori language, meaning 'land of the long white cloud'. While partly cloudy conditions can either enhance (by up to 25%, e.g. cumulus type) or reduce UV levels (Bodeker and McKenzie, 1996; McKenzie *et al.*, 1997), cloud cover in general reduces UV levels in New Zealand by about 25-30% per year (McKenzie *et al.*, 1996). Low amounts of aerosols and relatively small aerosol disturbances (Rosen *et al.*, 1997) result from the comparatively clean air in New Zealand. Apart from shifting the balance between direct and diffuse UV irradiance during summer (when aerosol concentrations are highest), in New Zealand effects of aerosols on UV levels (contrasting to the northern hemisphere) can in general be regarded as of minor importance (Forgan and Liley, 1998). Also contrary to the northern hemisphere, aerosols from the Pinatubo eruption showed no clear impact on ozone profiles over New Zealand (Forgan and Liley, 1998).

During the Austral spring of 1998, the largest-ever area of ozone depletion was recorded over Antarctica (27.3 million km^2), with the 'ozone hole' developing two weeks earlier than usual (NASA, 1998). Incidents of polar vortex ozone-depleted air passing over New Zealand can occasionally be observed (Brinksma *et al.*, 1998). Together with the decreasing background ozone levels and other factors, this could help explain the record low ozone levels measured over New Zealand during winter/early spring 1997 (Brinksma *et al.*, 1998). To date, however, these direct, vortex-related episodes are seen

to contribute only to a small degree to the overall reduction of ozone levels, when compared e.g. to the natural day-by-day variation in ozone of ca. 10% over New Zealand (McKenzie *et al.*, 1996).

Interestingly, the levels of the key ozone destroying species chlorine nitrate ($ClONO_2$), show a similar seasonal cycle to the ozone dynamics over New Zealand, also with the maximum and minimum levels occurring in early spring and autumn, respectively (Reisinger *et al.*, 1995). An annual trend of a 3-4% stratospheric increase in chlorine-containing reservoir species like HCl and $ClONO_2$ is considered possible at New Zealand latitudes (Reisinger *et al.*, 1995).

Past and future trends

During the last 25 years, ozone levels over New Zealand showed a decrease of ca. 3% per decade (Matthews, 1998). Erythemal UV levels during the 1980s increased by ca. 3-5% and plant-weighted UV at New Zealand southern latitudes increased by ca. 6-9% (Madronich, 1992). Much of the ozone reduction in New Zealand seems to have occurred during the mid 1980s, with smaller changes since. One explanation for the latter could be a delay in the break-up of the polar vortex due to decreased stratospheric temperatures, preventing the transport of ozone-rich air away from lower latitudes to the polar regions and thus even leading to a small increase in ozone over New Zealand during late winter/early spring (Matthews, 1998). Nevertheless, recent studies demonstrated conclusively that long-term decreases in summertime ozone over New Zealand have led to substantial increases in peak UV radiation intensities (McKenzie *et al.*, 1999).

Catalytic stratospheric ozone destruction due to increased halogen loading is predicted to continue well into the next century, but is perceived to decrease over the next decades as a result of international restrictions on halogen-containing source chemicals (McKenzie *et al.*, 1996). Estimates of the maximum ozone depletion vary depending on assumptions about compliance. If there was lasting compliance to the Montreal Protocol and its amendments, it is predicted that ozone depletion over latitudes covering New Zealand could peak early next century, with an 11% decline compared to 1960 levels

(Madronich *et al.*, 1995). This would result in a corresponding peak in erythemally-weighted UV of ca. 15%.

The New Zealand pasture ecosystem

From the above it is evident that the temperate pasture species in New Zealand experience much more pronounced UV-B irradiation compared to their origin at similar latitudes in the northern hemisphere. Today the predominant pasture species in New Zealand lowland areas are white clover (*Trifolium repens* L.) and ryegrass (*Lolium perenne* L.), grown in close association in mixtures (Harris, 1990). White clover is a herbaceous perennial legume, spreading by stolons with roots at nodes. The annual production of dry matter (DM) by ryegrass-clover based pastures ranges from 10-20 t DM/ha depending on summer rainfall and fertility (Coop, 1986), whereas the lower fertility pastures produce 5-10 t DM/ha. Pasture ecosystems in New Zealand can experience pronounced periods of summer drought, particularly in dry east cost regions (Campbell *et al.*, 1999).

In these pasture communities, white clover provides an important source of biologically-fixed nitrogen which drives ecosystem productivity. The white clover component of these pastures is a key component of the New Zealand pastoral industries which strongly rely on this low-cost feed source. In addition, white clover benefits pastoral industries by seasonal complementarity with grasses and its ability to increase livestock feed intake and utilisation rates (Caradus *et al.*, 1996). White clover fulfils these roles particularly well in countries with a moist, temperate climate such as New Zealand, where climatic conditions are especially suited for white clover growth (Brock *et al.*, 1989). The annual values for white clover nitrogen fixation in New Zealand are extremely variable depending on white clover content and availability of mineral nitrogen (Crush, 1987). However, it is estimated that the symbiosis with *Rhizobium* nitrogen fixing bacteria can provide around 200 kg N/ha/year, but has been measured in the range 17-400 kg N/ha/year.

Nitrogen fixation by legumes is the primary source of nitrogen for these pastures and little fertiliser nitrogen is applied. Nitrogen deficiency still limits agricultural production in New Zealand (Caradus *et al.*, 1996) and there are ongoing efforts to increase the

contribution of white clover to pasture through new breeding, pest management and grazing management strategies. These systems are grazed intensively by ruminant herbivores. This defoliation interrupts the pattern of plant development by regularly removing leaves before they are fully aged, altering source-sink relationships, changing canopy structure and influencing nutrient cycling. The grazing management generally removes a large proportion of the leaf area of the pasture plants, either under a continuous stocking system or with intermittent grazing allowing rest periods for re-growth. The predominant livestock enterprises on the lowland areas are dairying and lamb finishing, whereas the hill areas are predominantly used for sheep and beef production.

UV-B radiation and plants

History

Medical research on the effects of ultraviolet radiation on human skin 100 years ago inspired a range of studies on plant responses to UV over the last century. Early reviews of these findings were conducted by Popp and Brown (1936), and later by Lockhart (1961). Much of the UV-supplementation experiments during the first three quarters of that period were based on research with lamp systems emitting high levels of UV-C radiation (< 280 nm). These irradiation protocols were therefore more typical for extraterrestrial conditions. Nevertheless some of the earlier results were remarkably reflective of findings obtained from more current research using UV-B. For example a study on 67 plant species showed that Austrian Pine (*Pinus nigra*) needles sustained only slight injury after 44 consecutive days exposure to Martian-level UV (Cline and Salisbury, 1966). However, a number of herbaceous species, including red clover and other legumes were UV sensitive (Cline and Salisbury, 1966). It had been recognised for some time that numerous plant macromolecules (e.g. proteins and nucleic acids) absorb in the UV-B region (Giese, 1964). A range of adaptations and mechanisms against UV damage were suggested, including UV absorption by outer cell layers and formation of polyphenolics (Cline and Salisbury, 1966). UV-B-protective functions had been proposed for flavonoids in epidermal plant layers (Jagger, 1967).

A major methodological problem of the early studies was that measurements of UV radiation intensities were taken without consideration for the biological effectiveness of the wavelengths applied. Thirty years ago, however, Martyn Caldwell suggested the use of the generalised plant action spectrum, which defined a single curve for the relative photon effectiveness of UV-B for plant responses (Caldwell, 1968; Caldwell, 1971). This function was subsequently used in numerous examinations of UV-B-induced plant responses, strongly improving interpretation of measurements between studies. Since then there was an exponential increase in UV-B plant research, leading to the publication of more than 600 papers (Caldwell *et al.*, 1998).

More than 60 books and reviews have summarised this research, ranging from molecular to ecosystem levels (e.g. recently in Allen *et al.*, 1998; Britt, 1999; Caldwell *et al.*, 1998; Hader *et al.*, 1998; Huttunen *et al.*, 1998; Jansen *et al.*, 1998; Krupa *et al.*, 1998b; Laakso and Huttunen, 1998; Mackerness and Jordan, 1999; Mackerness and Thomas, 1999; Short and Neckles, 1999; Tevini, 1999; Tevini, 2000; Zepp *et al.*, 1998). A review book recently summarised UV-B effects on terrestrial ecosystems (Rozema, 1999, and chapters therein). UV-B effects on agriculture and agro-ecosystems have also been reviewed extensively (Campbell and Hofmann, 1998; Corlett *et al.*, 1997; Krupa and Kickert, 1993; Krupa *et al.*, 1998a; Krupa *et al.*, 1998b; Olszyk *et al.*, 1996; Rozema *et al.*, 1997c; Zinser *et al.*, 1997). Individual findings will be reviewed in more detail during the introductions for the individual Chapters of this thesis.

Legumes and white clover

It has been predicted that a primary consequence of increased UV-B in New Zealand would be a reduction in white clover growth rates to a greater extent than observed in grasses (Campbell *et al.*, 1999). Previous findings (Krupa *et al.*, 1998b; Teramura, 1990) identify legumes as one of the most UV-B-sensitive plant families. Both temperate and tropical legumes appear to be affected (Singh, 1997). Various effects of UV-B on legumes have been described, from molecular studies (Jordan *et al.*, 1994; Mackerness *et al.*, 1998; Strid *et al.*, 1996) to levels of growth (Antonelli *et al.*, 1997; Singh, 1997). However, other research suggests less distinctive effects on legumes (Deckmyn and Impens, 1995; Miller *et al.*, 1994). Sensitivity appears to be dependent on the particular species involved and environmental conditions (Antonelli *et al.*, 1997;

Cen and Bornman, 1990). Developmental stage of the tissue (Jordan *et al.*, 1994) and duration of UV-B exposure also seem to be important (Björn *et al.*, 1997; Deckmyn and Impens, 1995; Stephanou and Manetas, 1997). At higher UV-B levels, a white clover population in Jamaica showed decreases in growth (SLM) and UV-B-absorbing compounds, contrasting with other simultaneously investigated plant species (Rozema *et al.*, 1997a).

Studies on species of clover and ryegrass showed variable results regarding their UV-B sensitivity (Deckmyn and Impens, 1998; Kaiser, 1995; Krupa *et al.*, 1998b; Matthew *et al.*, 1996; Nakayama *et al.*, 1996; Newsham *et al.*, 1998; Papadopoulos *et al.*, 1999; Rozema *et al.*, 1997a). While there is some information available on UV-B effects on native New Zealand vegetation (Hunt, 1997; Hunt *et al.*, 1996), only one published study has so far described UV-B effects on the New Zealand pasture association (Matthew *et al.*, 1996). This will therefore be described in more detail in the following.

Using filters to reduce current UV-B radiation levels, the experiment tested effects of ambient UV-B levels on white clover and New Zealand pasture growth under sheep grazing (Matthew *et al.*, 1996). A UV-B-transmitting perspex sunbed filter and a UV-B-absorbing glasshouse polythene film were used to create contrasting UV-B environments. The UV-B-opaque canopy reduced ambient UV-B levels by about 87%, while the perspex filter absorbed ca. 25% of ambient UV-B. Both canopies reduced PPF levels by around 10-13%. PPF was ca. 3% higher and temperature was increased by 0.4°C under the transmitting canopy, compared to the absorbing filter.

Differences in growth parameters were not significant during the early harvest periods in spring and early summer but by mid-summer (when UV-B levels were highest) the UV-B-transmitting canopy decreased sward height and herbage mass by 14% and 19%, respectively. This was accompanied by a decline in ryegrass leaf extension rate under the UV-B-transmitting filter. During summer, ryegrass leaf elongation decreased over time, showing a non-significant trend two weeks after grazing and a significant decrease three to four weeks later. This was more pronounced in young tillers (-25%), compared to adult plants (-5%). A similar effect was observed on white clover leaf area and petiole elongation, showing decreases of 14% and 22%, respectively.

These observations are consistent with several other studies using methods of solar UV-B exclusion. For example, a reduction of ambient UV-B levels by 8-10% resulted in greater height and growth in bush bean in Portugal (Saile-Mark and Tevini, 1997). Using the ozone filter technique at a similar location, 10-35% lower growth was found in sunflower and maize seedlings at near ambient UV-B levels compared to a 30% reduced UV-B environment (Mark and Tevini, 1997). However, in other studies using differentially UV-transmitting filters in the Netherlands, no solar UV-B effects on biomass production, morphology and photosynthesis could be detected in four coastal grassland species (Tosserams *et al.*, 1996) or in *Vicia faba* seedlings (Visser *et al.*, 1997b).

In the New Zealand study, there was a trend suggesting that clover percentage in the sward was reduced in the UV-B-transmitting treatment but the generality of this finding was limited due to inherent constraints in the use of UV-B-transmitting canopies for this type of research. In particular, pronounced effects of the covering canopies were observed in terms of temperature, possibly leading to higher nitrogen levels, and thus increasing the longevity and content of ryegrass in the sward. This is supported by the simultaneously measured decrease in ryegrass leaf senescence under both covers. Nevertheless, the results of that study suggest that the present solar UV-B levels in New Zealand can have an effect on regularly defoliated New Zealand field swards, impacting on both white clover and ryegrass and possibly also on the interaction between the two species.

Population studies

White clover is a plant species that can conveniently be used to investigate within-species differences in stress responses due to extensive plasticity, on both the genotype and the population levels (Caradus, 1994; Caradus *et al.*, 1993a). Population comparisons are not only useful in examining the consistency of the UV-B response within a species but also allow the assessment of relationships between plant attributes and UV-B responses in plant types not confounded by species-specific differences. In the context of the present work, the term 'population' will include cultivated varieties (cultivars) and breeding lines as well as ecotypes collected in the wild.

If selection pressure as a result of increased UV-B radiation is severe, it is possible that the population structure of pastures may shift towards genotypes which are better adapted to higher UV-B levels. It has been suggested that a shift in population genetics may be most rapid in the clover populations (Campbell *et al.*, 1999). Due to the large intrinsic genetic plasticity within many developmental aspects of white clover it is reasonable to expect differential responses to UV-B among white clover populations. White clover has shown population differences in response to a number of other environmental variables, including drought (Wang *et al.*, 1996; Woodfield and Caradus, 1987), mineral supply (MacKay *et al.*, 1990), frost (Caradus *et al.*, 1989a) and aluminium stress (Caradus *et al.*, 1987).

Population-specific responses to UV-B have been demonstrated in a wide variety of plant species, for instance in birch (Lavola, 1998), *Brassica* (Allen *et al.*, 1997; Olsson *et al.*, 1998), *Coleus* (Burger and Edwards, 1996), cucumber (Middleton *et al.*, 1996), maize (Correia *et al.*, 1998; Correia *et al.*, 1999a), pea (Gonzalez *et al.*, 1996; Mepsted *et al.*, 1996), *Pinus* (Pukacki and Modrzynski, 1998), *Phaseolus* (Saile-Mark and Tevini, 1997), rice (Barnes *et al.*, 1993; Dai *et al.*, 1994; Kumagai *et al.*, 1999), *Silene* (van de Staaji *et al.*, 1997), soybeans (Murali *et al.*, 1988) and wheat (Hader, 1996). In soybeans, for example, studies suggest UV-B sensitivity for about two thirds of the examined populations (Biggs *et al.*, 1981; Teramura and Murali, 1986).

Underlying mechanisms

There are suggestions that origin from habitats high in ambient UV-B irradiation confers UV-B tolerance (Barnes *et al.*, 1987). Other studies, however, do not support this hypothesis. Rice cultivars from equatorial origin in general were not more tolerant to UV-B than those from higher latitudes (Dai *et al.*, 1994). Tropical plant species also can exhibit considerable sensitivity and it has been shown that current levels of UV-B already impact on low-latitude species (Searles *et al.*, 1995).

Furthermore, it has been proposed that tolerance to other forms of stress may confer UV-B tolerance (Al-Oudat *et al.*, 1998; Gwynn-Jones *et al.*, 1999b). Stress in the context of this thesis is defined as the extent to which external constraints limit dry matter production (Grime, 1979; Poorter and Garnier, 1999). A recent discussion of

potential UV-B effects on plant population genetics suggests that elite germplasm producing high yields may be more UV-B-sensitive, while UV-B tolerance in natural plant populations may be linked to smaller size (Groth, 1998). This would also be supported by ecological theory on stress tolerance, establishing a link between plant productivity (growth rates), habitat productivity and stress tolerance (Lambers and Poorter, 1992; Poorter and Garnier, 1999). According to this, a primary axis of adaptation separates 'competitors' (specialised primarily towards high rates of biomass acquisition in productive environments, but vulnerable to high rates of resource loss under stress) from 'stress-tolerators' showing lower productivity and higher resistance to biomass reduction in unproductive habitats (Grime, 1979; Grime *et al.*, 1997). This is also of particular relevance for plant functional types. These are sets of plants that exhibit similar attributes in response to environmental conditions (Diaz and Cabido, 1997). To date no studies are available that investigate the relationship between UV-B responsiveness and functional plant types (Gwynn-Jones *et al.*, 1999b). This is compounded by the fact that usually little environmental background information is available in most plant studies on UV-B responsiveness (Gwynn-Jones *et al.*, 1999b).

It is surprising that to date these relationships have not been investigated in detail for UV-B responsiveness. It is timely to test whether stress-tolerant populations are better protected against UV-B, and if so, how this could arise. In particular it can be proposed that slow-growing stress-tolerant plant types would be more tolerant to UV-B than faster-growing, productive plants. For the New Zealand situation, it is of importance to determine how the commonly used white clover cultivars bred for high productivity compare with ecotypes from natural, particularly stress-exposed, environments. Common cultivars are often relatively sensitive during periods of water stress and there are intraspecific differences in this response (Wang *et al.*, 1996).

In assessing genetic variation in UV-B tolerance there is a need to identify: (1) which attributes appear to make plants more susceptible or more tolerant/resistant to UV-B damage, and (2) which attributes appear to be the most responsive to enhanced UV-B exposure. Attributes often related to UV-B tolerance in experimental studies include content of UV-B absorbing compounds and anti-oxidants, leaf thickness, specific leaf area, architecture, and habitat from origins with naturally high UV-B background levels. Attributes most likely to be affected by elevated UV-B radiation in experimental studies

include growth, specific leaf area, content of UV-B absorbing compounds, palatability, stomatal conductance, leaf water content, and architectural parameters (Campbell and Diaz, 1998; Krupa *et al.*, 1998b; Rozema *et al.*, 1997b).

The use of multivariate statistical approaches, particularly principal components analysis (PCA), has been recommended for studies of numerous plant attributes across various plant species or populations (Grime *et al.*, 1997). Such multivariate analytical techniques are readily available in most statistical packages and allow combined analysis into a single data set which normally would require numerous univariate analyses of variance (Matthew *et al.*, 1994). In particular the usefulness of these approaches has been pointed out for summarising multivariate data sets in ecological investigations, e.g. of consequences due to climate change (Diaz and Cabido, 1997). PCA was used in the examination of species tolerance to extreme events (MacGillivray and Grime, 1995), showing that nutrient stress-adapted plant species also display greater resistance to drought and frost. Other studies used PCA to illustrate that the trade-off between high rates of resource acquisition and stress tolerance is linked to differential mineral nutrient accumulation (Grime *et al.*, 1997). A study of environmental adaptation within three populations in *Avena barbata* utilised PCA and discriminant analysis to show that the populations mainly differ in their adaptation to reduced water availability and to different light conditions (Somersalo *et al.*, 1998). PCA demonstrated greater tolerance to drought and frost in stress-adapted plant species (MacGillivray and Grime, 1995). PCA and cluster analysis were also used to classify white clover cultivars on the basis of leaf size and cyanogenesis (Caradus *et al.*, 1989b). However, despite their proven usefulness in ecological literature, there is a general lack of multivariate investigations in the examination of UV-B responses in plants.

Interaction with other factors

Drought

A key issue to be determined is whether effectiveness of increased UV-B radiation on pasture species would be altered by other environmental factors, such as nutrient or water stress. The necessity to investigate UV-B effects in conjunction with other stress factors including drought has been pointed out in a number of reviews (e.g. Bornman

and Teramura, 1993; Caldwell *et al.*, 1995; Jordan, 1996; Tevini, 1993). Findings from earlier studies (Murali and Teramura, 1985b; Murali and Teramura, 1986b; Sullivan and Teramura, 1990; Teramura *et al.*, 1984a; Teramura *et al.*, 1984b; Teramura *et al.*, 1983; Tevini *et al.*, 1983a) strongly suggested the need to study this interaction for species of the New Zealand pasture system, where the phase of high UV-B levels often coincides with periods of summer drought.

More than 20 studies have investigated the UV-B effects on plants in combination with differential water supply. This includes field-based research (Allen *et al.*, 1999; Barnes *et al.*, 1995; Björn *et al.*, 1997; Chaves *et al.*, 1997; Conner and Zangori, 1997; Drilias *et al.*, 1997; Manetas *et al.*, 1997; Murali and Teramura, 1986a; Murali and Teramura, 1986b; Nikolopoulos *et al.*, 1995; Petropoulou *et al.*, 1995; Stephanou and Manetas, 1997; Sullivan and Teramura, 1990; Teramura *et al.*, 1990) and indoor studies using glasshouses or controlled environment rooms (Balakumar *et al.*, 1993; Chaves *et al.*, 1997; Conner and Zangori, 1998; Eswaran *et al.*, 1993; Nogues *et al.*, 1998; Premkumar *et al.*, 1993; Schmidt *et al.*, 2000; Teramura *et al.*, 1984a; Teramura *et al.*, 1984b; Teramura *et al.*, 1983; Tevini *et al.*, 1983a).

UV-B has been shown to aggravate drought effects, e.g. for photosynthetic parameters or growth (Nikolopoulos *et al.*, 1995; Teramura *et al.*, 1984b; Tevini *et al.*, 1983a). Paradoxically, however, water stress may also reduce the damage caused by UV-B exposure, suggesting mutual amelioration of the stress forms by each other. For example, beneficial effects of the UV-B × drought stress combination have been described for anatomical and biochemical responses in cowpea (Balakumar *et al.*, 1993). In pea, UV-B was found to ameliorate the drought impact via reductions in leaf area, stomatal conductance and plant water-loss (Nogues *et al.*, 1998). Similar effects have been observed in several studies of *Pinus* and can be related to UV-B-induced morphological changes such as increased leaf cuticle thickness (Björn *et al.*, 1997; Manetas *et al.*, 1997). Higher leaf water content under the UV-B × drought combination in *Arabidopsis* has been related to biosynthesis of stress proteins and osmolytes (Schmidt *et al.*, 2000). In *Cistus*, not only a UV-B-induced stimulation in flavonoid production was found, but also a synergistic increase of this response under drought

(Chaves *et al.*, 1997). The production of flavonoids under these conditions is seen to contribute to stress protection.

Interaction of UV-B and drought might also alter within-species differences in UV-B sensitivity. Contrasting morpho-physiological responses to UV-B were observed in two soybean populations in dependency to moisture supply (Murali and Teramura, 1986b). While the cultivar 'Williams' remained unaffected, 'Essex' displayed decreases in yield, leaf area, CO_2 assimilation and stomatal resistance under adequate precipitation, but not in seasonal drought (Murali and Teramura, 1986b; Teramura *et al.*, 1990). These findings highlight the need to investigate mechanisms that could alter effectiveness of one stress form by another, e.g. changes in the levels of UV-B-absorbing compounds or osmoprotectants.

Duration of UV-B exposure

Furthermore, duration of UV-B exposure can also modify UV-B effectiveness. Studies in cucumber showed that increased time of exposure to UV-B can lead to detrimental effects for growth and photosynthesis (Tevini *et al.*, 1983a). Similarly cumulative effects have also been observed in heathland species of *Calluna* and *Vaccinium* (Björn *et al.*, 1997). Other findings, however, showed beneficial effects of time and plant development for UV-B sensitivity (Teramura *et al.*, 1984b; Visser *et al.*, 1997b).

Plant-herbivore relationships

It has been predicted that UV-B-induced reduction of white clover content in pasture ecosystems and resulting reduced nitrogen input would impact negatively on the forage production and forage quality of New Zealand pastures (Campbell *et al.*, 1999). Lower overall productivity as a result of lower nitrogen inputs would increase feed deficits for livestock, and result in a reduced stock carrying capacity. Potentially, a lower proportion of clover in the pasture can also modify grazing behaviour. In cases where there is a high clover content, higher utilisation of the grass component of the community can be achieved by grazing livestock. Changes in the biochemistry of plant tissues might also alter the nutritional value of the herbage per unit of dry matter. From other work it could be anticipated that protein, carbohydrate, fibre contents and

secondary plant chemical compounds may be altered in white clover as a result of higher UV-B radiation levels (Grant-Petersson and Renwick, 1996; Hatcher and Paul, 1994; Yazawa *et al.*, 1992).

Direct and plant-mediated indirect effects of UV-B on consumer organisms have been suggested for some time (Caldwell *et al.*, 1989). To date some studies have investigated UV-B effects on the plant-herbivore relationship (e.g. Grant-Petersson and Renwick, 1996; Hatcher and Paul, 1994; Lavola *et al.*, 1998; Rousseaux *et al.*, 1998; Salt *et al.*, 1998). Considering the impact of UV-B on secondary metabolism (Grant-Petersson and Renwick, 1996; McCloud and Berenbaum, 1994; Meijkamp *et al.*, 1999) such investigations seem warranted, especially for pasture plants like white clover where changes could influence secondary metabolism-mediated plant interactions with several trophic levels. This includes nitrogen fixation, attraction to pollinators, plant-herbivore interaction and forage quality (Dakora, 1995). Production of flavonoids and other phenolics in response to UV-B treatment has frequently been described and reviewed (Bornman *et al.*, 1997; Jordan, 1996; Meijkamp *et al.*, 1999). Such changes can also relate to protection of plants from increased levels of UV-B (Bornman *et al.*, 1997; Middleton and Teramura, 1993). UV-B-screening effects as well as radical-scavenging antioxidant functions and more effective energy dissipation have been proposed (Bornman *et al.*, 1997; Meijkamp *et al.*, 1999; Smith and Markham, 1998).

There is evidence that UV-B-induced phenolics such as flavonoids can act as feeding deterrents (Dakora, 1995; Grant-Petersson and Renwick, 1996), although other findings demonstrate that the involvement of plant phenolics in host plant resistance is less than conclusive (Bi *et al.*, 1997; Lavola *et al.*, 1998). UV-B may influence herbivory also through other changes in nutritive quality, e.g. alteration in levels of amino acids (Salt *et al.*, 1998), carbohydrates or nitrogen (Hatcher and Paul, 1994). In pea, it was shown that increases in leaf nitrogen concentration, rather than phenolics, determined herbivore response (Hatcher and Paul, 1994). UV-B-mediated changes in nitrogen production could be of particular importance for white clover populations depending on nitrogen-fixation for their nitrogen intake and producing nitrogenous compounds such as cyanogenic glucosides. Until now, no information has been available on the alteration of cyanogenesis in response to UV-B treatment. In some white clover populations, the presence of the cyanogenic glucosides linamarin and lotaustralin, and the structural gene

for the enzyme linamarase, can result in cyanogenesis in the leaf tissue. This production of cyanide in response to stress can deter feeding insects such as molluscs and possibly insects. Plants that possess only the cyanoglucosides, but not the enzyme, are protected against herbivores that have beta-glucosidases in their gut, e.g. molluscs (Kakes, 1989).

In June 1998, a field-based UV-B supplementation experiment was begun in Palmerston North, New Zealand to test some of the predicted effects of increased UV-B radiation on an established ryegrass and white clover (cultivar 'Huia') pasture. Three supplemental UV-B radiation treatments are being used, representing 0, 15 and 30% reductions in annual ozone concentration. These correspond to predicted clear-sky summer maximum increments in plant-weighted UV-B doses at the Palmerston North site of 0, 30 and 72% respectively, according to model predictions (McKenzie, 1991). Preliminary results from this experiment illustrate that detectable changes are occurring in the growth and development of species within the pasture. For example, measurements of cyanogenesis conducted by the thesis author in spring 1998 using a picrate paper test (Williams *et al.*, 1998) indicate a rise in cyanogenesis in leaves of field-grown 'Huia' with increasing exposure to elevated UV-B radiation (Fig. 1-1). This response is consistent with higher stress on the plant population, and provides an early indication of changes which might impact on herbivory by invertebrates. Shifts in plant-herbivore interactions may in turn directly or indirectly (via trophic cascades) alter community and ecosystem dynamics. Very few studies, however, have addressed effects of UV-B radiation on trophic interactions, and none have been conducted with species of importance to New Zealand agriculture.

The need for research activities in a number of areas outlined above is in several aspects also reflected by earlier recommendations, urging research on plant species introduced into New Zealand agro-ecosystems (Laing, 1991; Laing, 1993; McKenzie, 1992), including: (1) investigations on New Zealand pasture species, (2) estimates of the degree of UV-B sensitivity in these species, (3) determination of the degree of variability in UV-B sensitivity, (4) examinations of mechanisms of UV-B action, (5) determination of the metabolic costs of UV-B protection, and (6) determination of UV-B effects on product quality (e.g. flavour).

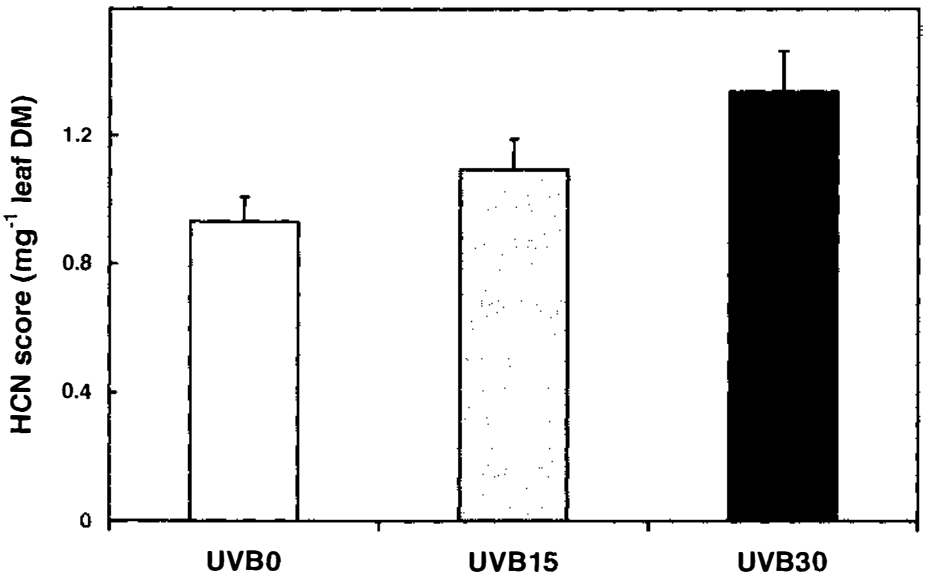


Fig. 1-1. Intensity of cyanide (HCN) production in the white clover cultivar 'Huia' growing at three UV-B levels in a grazed pasture community in Palmerston North, New Zealand (September 1998). The bars are the standard errors of the means. UVB 0, UVB 15 and UVB 30 correspond to UV-B levels at 0, 15% and 30% ozone depletion, respectively. DM = dry mass. Figure published in Campbell *et al.* (1999).

General description of the experimental approaches

All experiments used growth rooms at the National Climate Laboratory in Palmerston North, New Zealand with average PPF levels of 420 to 435 μmol m^{-2} s^{-1} and plant-weighted UV-B levels of 13.3 kJ m^{-2} d^{-1}, mimicking 25% summer ozone depletion above Palmerston North. All experiments were conducted on a population level to (1) determine the generality of possible UV-B responses within white clover, (2) expose genetic differences among white clover populations in response to UV-B, and (3) to discern possible functional mechanisms underlying differential UV-B responsiveness. A number of white clover populations with a wide range of genetic and geographical backgrounds were used in the experiments. This comprised populations collected in the wild (ecotypes), including high latitude populations from Scandinavia, low latitude populations from South America as well as mid latitude (temperate) populations from Asia, Southern Europe and New Zealand. Other populations used were productive agricultural cultivars selected for high agronomic value, breeding lines with ornamental

leaf characteristics and an octoploid line with double the usual tetraploid white clover chromosome set. Detailed description of the white clover populations used can be found in Chapter 2 (see Table 2-1).

These investigations included examinations of other biotic and of abiotic interactions. This involved interaction with drought, time and insect species-specific herbivory as well as UV-B susceptibility of frequently defoliated plants. A number of morphological and physiological investigations were conducted, including attributes linked to photosynthetic function, nutritive value and insect performance as well as morphogenetic features of bud and leaf structure (Fig. 1-2). A subset of these attributes represented those that could be particularly important for UV-B protection (Fig. 1-2). It was anticipated that UV-B effects on these features would eventually be reflected in morphological changes and altered productivity (Fig. 1-2).

Aims

In particular these studies intended to test the following hypotheses:

1. White clover is sensitive to UV-B under controlled environmental conditions, based on total plant growth, morphology and physiology.
2. These responses differ among white clover populations.
3. Differences in UV-B sensitivity at the population level can be linked to plant productivity.
4. UV-B responsiveness is related to other constitutive and UV-B-induced features of white clover morphology or physiology.
5. UV-B tolerance can be linked to habitat adaptation, particularly tolerance to other forms of stress.
6. UV-B responsiveness is modified by drought.
7. UV-B effectiveness changes over time.
8. UV-B responsiveness can be detected in frequently defoliated white clover leaves.
9. The performance of insect herbivores differs when reared on UV-B-treated white clover foliage.

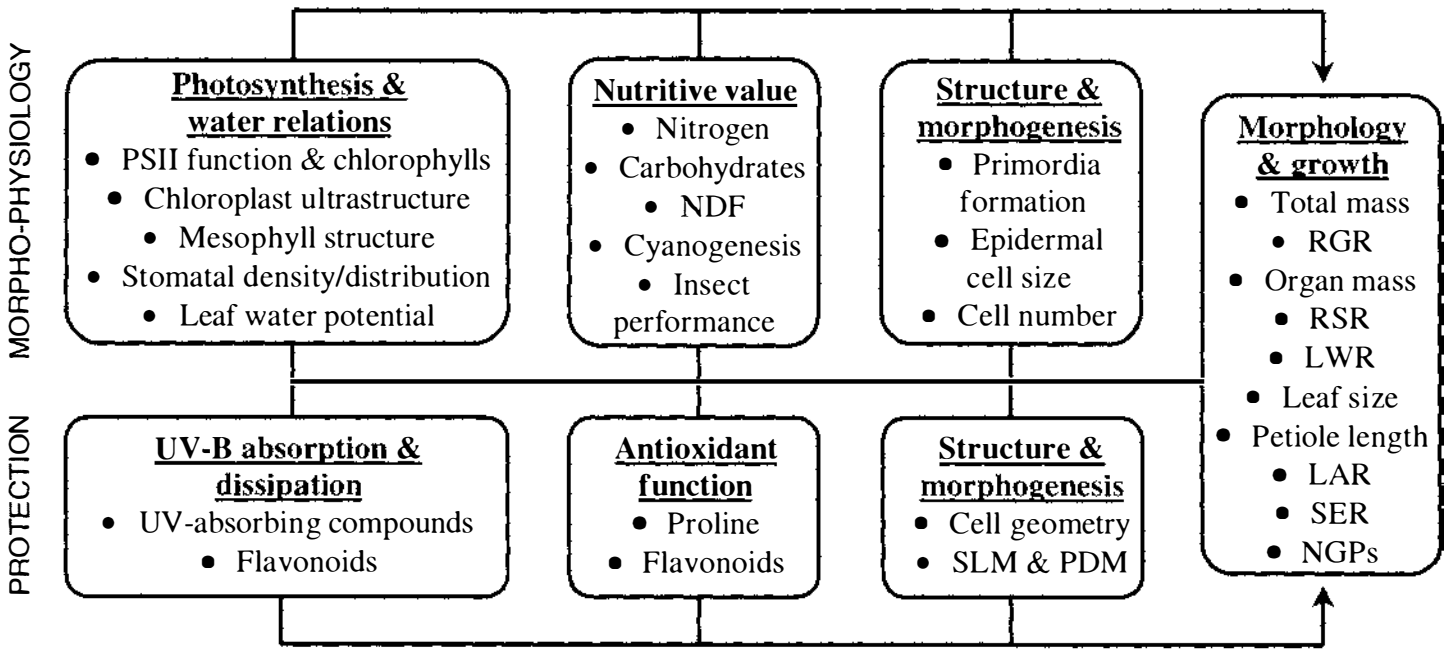
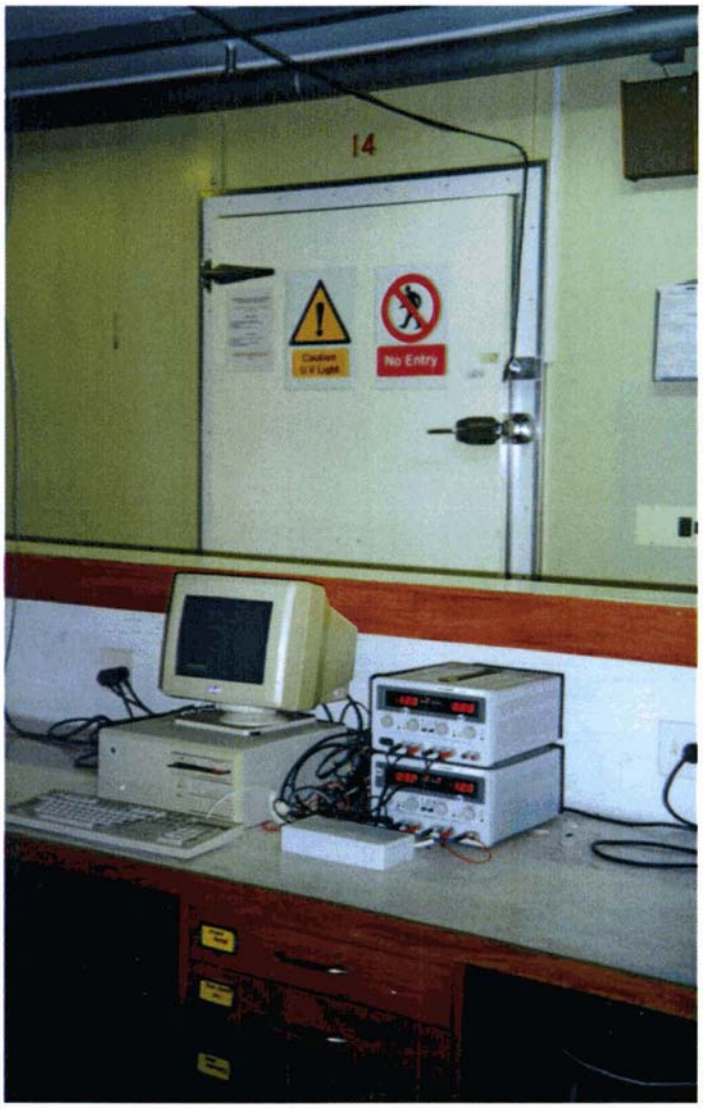


Fig. 1-2. Scheme of the current research project, based on morphological and physiological attributes in white clover. LAR = leaf appearance rate; LWR = leaf weight ratio; NDF = neutral detergent fibre; NGPs = number of growing points; PDM = percent leaf dry mass; PSII = photosystem II; RGR = relative growth rate; RSR = root:shoot ratio; SER = stolon elongation rate; SLM = specific leaf mass.

Chapter 2. *Trifolium repens* responses to ultraviolet-B radiation: integrated analysis of 17 traits and 26 populations



UV-B screening facilities: computer feedback system and walk-in growth chamber

An abbreviated version of this Chapter has been submitted to *Science*.

Abstract

The pasture legume white clover (*Trifolium repens* L.) is experiencing increased levels of ultraviolet-B radiation (UV-B) in New Zealand due to depletion of the stratospheric ozone layer. This study analysed the consequences of UV-B supplementation on 17 morphological, morphogenetic and physiological attributes of *T. repens* to identify traits linked to the UV-B response. Principal components analysis (PCA) was conducted on 26 *T. repens* populations to explore within-species variation in this response. After 18 days of exposure to 13.3 kJ m^{-2} d^{-1} UV-B in controlled environments, UV-B significantly decreased total plant dry mass (-26%) with reductions in leaves, stolons and roots. A less pronounced UV-B effect on roots led to a 7% increase in the root:shoot ratio, concomitant with a 2% decrease in the leaf weight ratio. Reductions were also observed in relative growth rate (-14%), number of growing points (-9%) and petiole length (-22%). Differential decreases in leaf size and leaf dry mass resulted in an 8% increase of the specific leaf mass. Adaxial epidermal cell size was reduced by 10% and stomatal density was increased by 11%. UV-absorbing compound levels increased slightly by 3% and intrinsic efficiency of photosystem II displayed a small overall reduction by 2% in response to UV-B. Aspects of cell division and cell expansion were negatively affected by UV-B. The main PCA dimension of UV-B sensitivity in *T. repens* (PC1) was characterised by changes in whole plant growth attributes, young leaf growth and, to a lesser degree, UV-absorption. UV-B-induced alterations in allocation and partitioning within and between plant organs constituted a secondary tier of UV-B responsiveness (PC2). Overall, UV-B tolerance was more easily predicted by traits linked to features of plant and habitat productivity, rather than by the likely UV-B level in the habitat of origin. Plant attributes related to UV-B tolerance included lower relative growth rate and smaller epidermal cell size as well as higher UV-B-induced accumulation of UV-absorbing compounds. Ecotypes from environments exposed to other forms of stress ranked highly for UV-B tolerance in the multivariate assessment, while several agricultural cultivars exhibited greater overall UV-B sensitivity. These results suggest differences in UV-B sensitivity depend on ecological specialisation with particular advantages for stress-adapted populations that have not been selected for high yield.

Introduction

Depletion of the stratospheric ozone layer is leading to increased levels of ultraviolet-B radiation (UV-B, 290-315 nm) reaching tropospheric levels in New Zealand (McKenzie *et al.*, 1999). Numerous responses to UV-B have been observed for plants, affecting molecular, physiological, morphological and ecosystem levels (e.g. reviewed more recently in Caldwell *et al.*, 1998; Mackerness and Jordan, 1999; Rozema, 1999; Tevini, 2000). Morphological changes frequently observed in response to UV-B irradiation include decreases in productivity, plant biomass and leaf area (Correia *et al.*, 1999b; Li *et al.*, 1998), increases in leaf thickness and specific leaf mass (Cen and Bornman, 1990; Cen and Bornman, 1993) as well as changes in biomass allocation (Gwynn-Jones and Johanson, 1996; Li *et al.*, 1998). UV-B-induced differences in leaf surface morphology have also been reported, including changes in the number and size of epidermal and stomatal cells (Staxen and Bornman, 1994; Stewart and Hoddinott, 1993; Tevini *et al.*, 1983b; Visser *et al.*, 1997a). There are also reports on UV-B-elicited decreases in the intrinsic efficiency (F_v/F_m) of photosystem II (PS II), indicating inhibition of photosynthetic function in PS II (Zeuthen *et al.*, 1997). Frequently, UV-absorbing compound levels are increased in response to UV-B (Day and Demchik, 1996; Jordan *et al.*, 1998). This is considered to be a potential mechanism of protection as these compounds can act as selective optical filters (Murali and Teramura, 1986c).

White clover (*Trifolium repens* L.) is an important forage legume in temperate regions of the world, constituting high quality feed while efficiently providing nitrogen to pastures (Brock *et al.*, 1989). *T. repens* has shown a wide variety of plasticity, on both the genotype and the population levels (Caradus, 1994). It could be expected that variation might exist among *T. repens* populations with respect to sensitivity to UV-B. Several previous studies have reported differences in morphological and physiological UV-B responses among populations of a species, (e.g. Barnes *et al.*, 1993; Correia *et al.*, 1999a; Dai *et al.*, 1994; Murali and Teramura, 1986c). These differences might also be used to suggest possible underlying mechanisms of UV-B sensitivity.

Some reports examining the ambient UV-B radiation environment in which plants grow indicate higher susceptibility for plants from lower natural UV-B backgrounds (Barnes *et al.*, 1987; Sullivan *et al.*, 1992). There are other indications that UV-B sensitivity

could be a function of intrinsic susceptibility to other forms of stress, for instance to drought (Al-Oudat *et al.*, 1998). Tolerance to one form of stress due to adaptation to another has also been demonstrated in other studies. For example, principal component analysis of 26 traits in 21 British plant species revealed greater tolerance to drought and frost for nutrient stress-adapted species (MacGillivray and Grime, 1995). Similar multivariate analytical approaches have also been used in other investigations on stress tolerance (Grime *et al.*, 1997; Somersalo *et al.*, 1998). However, despite their proven usefulness in ecological literature, there is a general lack of multivariate investigations in the examination of UV-B responses in plants. This appears particularly relevant for investigations of UV-B sensitivity based on numerous traits and several populations within a species.

This study analysed 17 morpho-physiological characteristics of 26 *T. repens* populations grown with and without supplementation of UV-B radiation. The UV-B response for each trait and each population was established from the difference between treatment and control values and the resulting population × response matrix was subjected to principal components analysis. Attributes included aspects of plant size and growth, measurements of biomass allocation, shoot and leaf morphology, leaf surface characteristics, intrinsic photochemical efficiency of PS II and concentration of UV-absorbing compounds. The objective was to determine the extent of changes for these attributes in *T. repens* and, as a consequence, to obtain a summary of the multivariate effects characterising the overall UV-B response. Numerous populations of *T. repens* with a variety of original habitats were used to explore whether they might appear dissimilar in their overall UV-B sensitivity. From this it was intended to analyse which plant features would distinguish UV-B-tolerant from sensitive *T. repens* populations. It was hypothesised that stress-adapted populations or those from an origin of higher background UV-B levels would be more tolerant to UV-B.

Materials and Methods

Experimental design

The experimental design was a 2 × 26 factorial, with two levels of UV-B radiation and 26 populations of *T. repens*. There were ten replicated pots per population in each UV-B treatment. Pots were arranged randomly on trolleys which were rotated in each room every second day to reduce effects from differential shading under the UV-B rigs.

Plant cultivation and UV-B irradiation

T. repens seeds were obtained from the Margot Forde Germplasm Centre in Palmerston North, New Zealand. After scarification, seeds were placed in petri dishes on moist filter paper and incubated at 25°C for 3-5 days. The emerging seedlings were transferred to trays containing watered potting mix and placed in a glasshouse at a temperature of 20°C. After four weeks, the seedlings were transplanted to individual 1.5 L pots in the same medium. Eight weeks later, four stolon cuttings were taken from each pot and planted pair-wise in sterilised sand/mineral mix at opposite corners of square 3 L pots. Pots were placed in growth rooms of the National Climate Laboratory (Palmerston North) and supplied three times daily for 2 min with an automatic drip irrigation system using half-strength Hoagland's nutrient solution. The average photosynthetic photon flux (PPF) during the 10 h light period was 435 μmol m^{-2} s^{-1}, supplied by a water-screened array of four 1 kW Sylvania 'metal-arc' high pressure discharge lamps, together with four 1 kW Philips tungsten iodide lamps. PPF was measured using a LI-COR 185 meter with attached quantum sensor. Day/night room temperatures were 18°C and 13°C. The day/night values for relative humidity were 80% and 73% and those for mean CO_2 levels 370 ppm and 390 ppm.

After eight weeks, pots were thinned down to one plant per pot and 10 representative stolons from each population were used to determine initial plant dry mass. The remaining plants were allowed to grow without supplemental UV-B for a further 10 days before onset of the UV-B treatment. The level of irradiation was 13.3 kJ m^{-2} d^{-1} of plant-weighted, biologically effective UV-B (Caldwell, 1971), normalised to 300 nm.

This dose corresponds to a 25% depletion of summer ozone levels above Palmerston North (latitude: 40.2 S, longitude: 175.4 E). UV-B radiation was supplied 8 hours per day for $2^1/_2$ weeks in the middle of the photo-period from Philips TL 40W/12 RS UV fluorescent tubes, enclosed in cellulose diacetate filters. The tubes were suspended 500 mm above plant level. A Solar Light Company model 501A UV biometer sensor measured the UV output which was controlled by a feedback system to maintain the constant, pre-set UV-B irradiation doses in response to tube ageing and cellulose acetate filter degradation. UV-B levels were monitored and recorded digitally with an IBM-PC computer that adjusted an analogue voltage supplied to Osram high-frequency dimming ballasts to achieve the required UV-B output from the fluorescent lamps. Cellulose diacetate filters were changed when the feedback system output approached maximum voltage. A Bentham DM150 UV spectroradiometer was used to calibrate the sensor at regular intervals against the generalised plant action spectrum (Caldwell, 1971).

Plant material

Twenty-six *T. repens* populations with a wide range of genetic and geographical backgrounds were included in the experiments (Table 2-1). Sixteen populations were ecotypes, i.e. populations collected in the wild. They included high latitude populations from Scandinavia, low latitude populations from South America as well as mid latitude (temperate) populations from Asia, Southern Europe and New Zealand. A further eight populations were agricultural cultivars, selected for high agronomic value in breeding programmes. Two further populations, 'Green' (higher chlorophyll levels) and 'Lime' (lower chlorophyll levels) represented unmultiplied selections (breeding lines) of the cultivar 'Huia'.

Table 2-1a. Site characteristics of 16 *T. repens* populations collected in the wild (ecotypes), arranged on a regional basis by latitude. Accession numbers are from the Margot Forde Germplasm Centre in Palmerston North, New Zealand.

			Site characteristics				
N°	Accession	Name	Country	Latitude	Altitude (m)	Annual rainfall (mm)	Other descriptors
1	C15133	Kangos [a]	Sweden	67.3°N	270	500	Sand with clay, abandoned cultivation, north of arctic circle.
2	C15121	Koskenkylä [a]	Finland	66.3°N	100	500	Sand with clay, cultivated land, near arctic circle.
3	C15130	Häggås [a]	Sweden	64.2°N	500	700	Southwest clay slope, abandoned cultivation.
4	C7618	Sacile [b]	Italy	45.5°N	100	1000	Open road verge, likely ladino type[i] with relative climatic tolerance but lower adaptation to sustained drought.
5	C9507	Veneto [b]	Italy	45.5°N	250	1000	Shady road verge in forest, likely ladino type[i] with relative climatic tolerance but lower adaptation to sustained drought.
6	C8749	Tienshan [c]	China	43.0°N	2000	600	Hay meadow, heavy winter snow, very cold.
7	C10541	Gudauri [d]	Georgia	42.3°N	2390	1200	Alpine meadow, western slope, moderate grazing pressure, cold.
8	C10523	Schrosha [d]	Georgia	42.1°N	275	500	Fallow with shrubs, southeast slope, light grazing pressure.
9	C10525	Sviri [d]	Georgia	42.1°N	155	900	Pasture and trees on flat, light grazing pressure.
10	C8734	Sarikamis [d]	Turkey	40.2°N	2300	580	Basaltic, extended snow cover and clouds, very cold.
11	C7986-C8022	Kahumingi [e]	New Zealand	40.5° S	200	615	North slope, dry hill country pasture, relatively high soil cation levels and lower organic matter %, plant genetic background most likely from cv. 'Huia'[i], relatively tolerant to nematode damage compared to 'Kavala' (but lower than 'Huia').
12	C8240-C8270	Kavala [e]	New Zealand	40.5° S	375	1400	South slope, wet hill country pasture, relatively low soil cation levels and higher organic matter %, plant genetic background most likely from cv. 'Huia'[i], prone to nematode damage compared to 'Kahumingi'.
13	C13286	Irazu [f]	Costa Rica	9.5°N	ca. 2000	ca. 1750	Roadside, tall type.
14	C6840	Colombian [g]	Colombia	ca. 5.0°N	n/a[g]	n/a	n/a.
15	C16028	Tulcan [h]	Ecuador	0.5°N	3200	>2000	Highland pasture with low clover content (1%), acidic peat soil with high Al (pH 4.5), mean annual temp approx. 11°C, low grazing pressure.
16	C16029	Sanana [h]	Ecuador	0.1°S	3600	>1500	Sloping open highlands pasture with sporadic clover content, acidic volcanic soil (pH 5), mean annual temp approx. 10°C, moderate grazing pressure.

[a] (Nordiska Genbanken, 1993); [b] (Forde and Easton, 1986); [c] Field notes Margot Forde China field collection expedition 1987; [d] (Caradus and Forde, 1996); [e] (Caradus *et al.*, 1990b); [f] Field notes from Costa Rica field collection (pers. comm. Paul Mueller); [g] 'Colombian' site description data not available (n/a); [h] Field notes from Ecuador field collection (pers. comm. Alan Stewart); [i] Derek Woodfield (pers. comm.)

Table 2-1b. Characteristics of 10 *T. repens* populations selected or bred for particular traits (cultivars and breeding lines). Accession numbers are from the Margot Forde Germplasm Centre in Palmerston North, New Zealand.

N°	Accession	Name	Country	Type	Description
17	C5067	Aran [j]	Ireland	Cultivar	Bred from French and Israel germplasm, high yielding, performs well in hay sward, less tolerant to grazing, some disease resistance.
18	C7545	Feathermark [j]	New Zealand	Cultivar	Similar genetic background and agronomic performance to 'Huia', red (anthocyanin-rich[k]) midrib leaf mark.
19	C13173	Haifa [j]	Israel/ Australia	Cultivar	Bred from Israel germplasm, high yielding, heat- and salt-tolerant, more sensitive to frost and drought, decreased persistence under grazing, relatively poor disease resistance, good performance in lowland conditions.
20	C7544	Huia [j]	New Zealand	Cultivar	Long-established New Zealand general purpose cultivar, selected for wide adaptation and yield, high phenotypic plasticity, persistence to grazing, high amino acid content, sensitive to P-deficiency, water stress and frost, relatively poor disease resistance, best suited for lowland conditions.
21	C13176	Kopu [j]	New Zealand	Cultivar	Includes germplasm from ladino cultivars, 'Pitau' (and thus 'Huia') and a Spanish strain, bred for lowland farming, nematode resistance and yield, more productive and leaves more upright habit than 'Huia', recommended for grazing, large roots but no persistence in dry hill country, frost sensitive.
22	C8761	Luclair [j]	France	Cultivar	Leaf mark discolouration and patch of anthocyanin[l], medium frost tolerance and forage yield, better drought resistance than 'Huia'.
23	Cl 1678	Prestige [j]	New Zealand	Cultivar	Bred from ecotypes in Northland (northern New Zealand), higher productivity than 'Huia', improved heat and drought tolerance, grazing-tolerant, suited for hill country climate and dry sunny slopes, relatively good disease resistance.
24	C11697	Sustain [j]	New Zealand	Cultivar	Includes Mediterranean and US germplasm, highly stoloniferous and persistent, well suited for grazing.
25	C15741	Green [m]	New Zealand	Breeding line	Genetic background from 'Huia', dark green phenotype selected for high chlorophyll content.
26	C15740	Lime [m]	New Zealand	Breeding line	Genetic background from 'Huia', lime green phenotype selected for low chlorophyll content.

[j] (Caradus, 1986) and (Caradus and Woodfield, 1997); [k] (Corkill, 1963); [l] (Lenoble and Papineau, 1970); [m] Description of 'Lime' and 'Green' from Derek Woodfield (pers. comm.)

Whole plant growth measurements

At harvest, aerial plant parts were collected and stored in plastic bags under darkness at 4°C before separation into stolons and leaves. After removal of the pots, roots were washed and separated from sand. All plant parts were dried at 80°C for 48 h. Allocation parameters calculated were root:shoot ratio (RSR, the ratio of root mass over shoot mass) and leaf weight ratio (LWR, the ratio of leaf mass over total biomass). Relative growth rate (RGR) per day was calculated from initial and final total plant dry mass. The number of growing points was obtained by counting the number of stolon tips present on each plant at final harvest.

Leaf measurements

Leaf size (area per leaf) of four fully open distal leaves per plant was measured at the end of the experiment with a LI-COR model 3100 area meter. Laminae were subsequently dried at 80°C for 48 h for dry mass determination. Specific leaf mass (SLM) of the laminae was calculated as the ratio of dry mass over leaf size. In addition, petiole length on the fully open distal leaf was measured at the end of the UV-B treatment period. Chlorophyll fluorescence measurements were conducted at room temperature on fully open distal leaflets of five replicate samples for each population under each UV-B level (Greer, 1995b). Immediately after collection, samples were stored on moist filter paper in cuvettes sealed with a rubber stopper and dark-adapted for 30 min. Measurements were carried out using a PAM fluorometer (Pam 101; Walz, Effeltrich, Germany). Initial fluorescence in darkness F_o was measured using a weak measuring light. Maximum chlorophyll fluorescence in darkness F_m and the fluorescence ratio F_v/F_m ($F_v = F_m - F_o$), indicating intrinsic efficiency of photosystem II (PS II) were determined using a flash intensity of 10 000 µmol m^{-2} s^{-1} (KL1500; Walz).

Methanol-extractable UV-absorbing compounds were estimated following standard procedures (Mirecki and Teramura, 1984). Four fully open distal trifoliate laminae were oven-dried and 15-20 mg of ground material was weighed and extracted in 1.4 ml MeOH:H_2O:HCl (79:20:1). Extraction in darkness for 24 h under occasional shaking was followed by centrifugation and spectrophotometric analysis of the supernatant in 3 ml quartz cuvettes. Absorbance readings at 300 nm were calculated on the basis of leaf

dry weight. This procedure was repeated on five replicates for each *T. repens* population under each UV-B treatment.

Epidermal studies

Samples were collected using a modified leaf replica technique (Beerling and Chaloner, 1992; Hilu and Randall, 1984). Using a fine brush, a solution of 4% formvar in chloroform (w/v) was applied to the middle leaflet of fully open distal *T. repens* leaf laminae. Celluloid tape was used to remove the dried solution, representing a cast replica of the epidermal surface. After placement on a glass slide, the replica was viewed through a Nikon Diaphot inverted microscope with a phase contrast Nomarski filter attachment. The image was then captured with a Sony black and white camera, connected to a Zeiss Kontron-VIDAS image analysis system, giving a resolution of 760 × 568 pixels. Digital image processing used VIDAS and MATLAB software.

Two frames were obtained from each of 10 leaf samples per population under each UV-B level. Stomata were identified manually in each frame and the number of stomata was determined in order to calculate stomatal density. The remaining epidermal cells in the frame were segmented by binary thresholding, followed by dilation until cells touched. After removal of stomata, the pixel area of epidermal cells inside a guard frame of defined area was determined. This value was divided by the number of epidermal cells examined to obtain mean epidermal cell size and the result was converted from pixels to μm^2. Stomatal density was calculated as the number of stomata per mm^2.

Statistical analysis

Univariate analyses and regression studies were conducted with the General Analysis of Variance and Regression Analysis procedures in GENSTAT (Genstat, 1993). Principal components analysis (PCA) (Kershaw and Looney, 1985) was used as an exploratory tool in order to indicate an overall measure of UV-B sensitivity for the 26 *T. repens* populations on the basis of the simultaneous analysis of 17 responses to UV-B. The analysis was performed with the SAS statistical package (SAS, 1996). PCA was chosen as this multivariate approach is considered to be particularly suitable for datasets with possible correlation among attributes (Manly, 1994). Correlations among variables are

removed by PCA and converted into sets of independent attributes. Thus, information from the individual responses is brought together to create new, combined variables representing uncorrelated vectors in 17 dimensions. The resulting principal components (PCs) are ranked in the PCA according to their importance in explaining the variation in the data set.

Data were log-transformed and the differences between means (representing measures of UV-B responsiveness) were subjected to PCA. Following PCA, these differences were regressed against the PC scores. The resulting probability values and correlation coefficients facilitated distinction of key traits characterising the UV-B response in each dimension. *T. repens* populations placed close to either end of a particular axis could then be related to responses positively or negatively correlating with a particular PC axis. A further aim was to identify constitutive plant attributes (i.e. measured under no UV-B supplementation) linked to the principal UV-B sensitivity measure (PC 1). To this purpose, PC1 scores were regressed against the constitutive means of the attributes. Finally, this was compared to regressions of PC 1 with the means of the attributes measured under UV-B supplementation.

Results

Univariate UV-B responses

Across populations, supplemental UV-B decreased plant dry mass in leaves, stolons and roots (Table 2-2). A slightly smaller relative reduction in root dry mass led to an increase in the root:shoot ratio and a small decrease in the leaf weight ratio. Relative growth rate, number of growing points and petiole length were also reduced under UV-B. Leaf size and leaf dry mass decreased differently under UV-B, leading to an increase in the specific leaf mass (Table 2-2). Leaf surface characteristics were also affected, including reduced epidermal cell size and increased stomatal density. There was a small increase in UV-absorbing compound levels and a slight decrease in dark-adapted chlorophyll fluorescence ratios (F_v/F_m) (Table 2-2).

Table 2-2. UV-B-induced univariate percent changes (calculated from the ratio UV+/UV-) in 17 plant attributes for *T. repens*, ranked by the degree of change. $P < 0.001$ = ***; $P < 0.01$ = **; $P < 0.05$ = *; $P < 0.10$ = $^{+}$.

Trait description	% *Change*	*P*
Adaxial stomatal density	+10.7	***
Specific leaf mass	+7.6	***
Root:shoot ratio	+6.5	*
UV-absorbing compounds	+3.2	$^{+}$
Ratio adaxial:abaxial stomata	+1.1	
Chlorophyll fluorescence	-1.8	***
Leaf weight ratio	-2.3	***
Number of growing points	-9.1	**
Adaxial epidermal cell size	-10.4	***
Relative growth rate	-14.2	***
Plant root dry mass	-20.7	***
Dry mass of first open leaf	-21.3	***
Petiole length	-22.4	***
Plant stolon dry mass	-23.5	***
Total plant dry mass	-25.8	***
Size of first open leaf	-27.0	***
Plant leaf dry mass	-27.9	***

Plant trait responses in the first principal component

The first principal component (PC1) explained 33% of the variance in the dataset, more than double to the next principal component. There were ten correlations, all of which could be found on the positive PC1 axis (Fig. 2-1 and Table 2-3). High scores on PC1 were characterised by large decreases in all whole plant biomass attributes, followed by young leaf lamina growth traits. This was accompanied by reductions in the number of growing points and adaxial epidermal cell size. In addition, the changes in PC1 tended to be associated with changes in the concentration of UV-absorbing compounds.

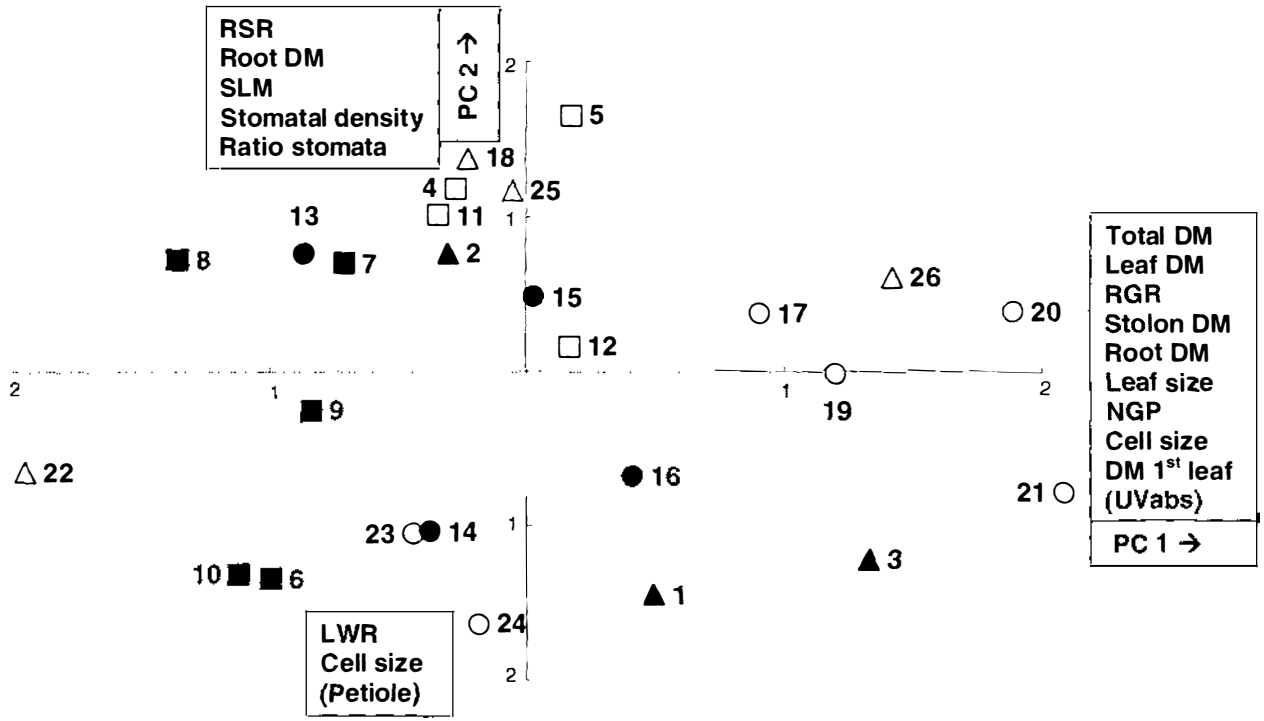


Fig. 2-1. PCA of 17 UV-B responses in 26 *T. repens* populations with PC1 scores plotted against PC2 scores. Associated plant responses are grouped at the end of each principal component. Abbreviations for plant responses: DM = dry mass; DM 1st leaf = DM of fully open distal leaf; LWR = leaf weight ratio; NGP = number of growing points; PC = principal component; Petiole = petiole length; Ratio stomata = ratio adaxial:abaxial stomata; RGR = relative growth rate; RSR = root:shoot ratio; SLM = specific leaf mass; UVabs = UV-absorbing compounds. Dark-adapted photochemical efficiency of photosystem II (F_v/F_m) remained the only response not associated with either principal component. Numbers identifying the individual populations are given in Table 2-1. Symbols for populations represent Asian (■), tropical (●), Scandinavian (▲), Mediterranean/New Zealand ecotypes (□), common cultivars (○) and populations with particular features of pigmentation (△).

Table 2-3. Correlation coefficients and probabilities for plant attributes linked to the first two principal components, ranked by probabilities. $P < 0.001$ = ***; $P < 0.01$ = **; $P < 0.05$ = *; $P < 0.10$ = $^{+}$.

Trait description	Correlation with PC1		Correlation with PC2	
	r	*P*	*r*	*P*
Total plant dry mass	0.952	***	0.127	
Plant leaf dry mass	0.925	***	-0.071	
Relative growth rate	0.879	***	0.096	
Plant stolon dry mass	0.778	***	0.288	
Plant root dry mass	0.717	***	0.575	**
Leaf size	0.691	***	-0.174	
Number of growing points	0.626	**	-0.015	
Adaxial epidermal cell size	0.610	**	-0.416	*
Dry mass first open leaf	0.590	**	0.121	
UV-absorbing compounds	0.332	$^{+}$	0.189	
Root:shoot ratio	-0.046		0.798	***
Specific leaf mass	-0.125		0.622	**
Leaf weight ratio	0.163		-0.620	**
Adaxial stomatal density	-0.294		0.481	*
Ratio adaxial:abaxial stomata	-0.179		0.458	*
Petiole length	0.320		-0.338	$^{+}$
Chlorophyll fluorescence	0.091		0.220	

Population responses in the first principal component

T. repens populations sensitive to UV-B (displaying stronger UV-B-induced responses in PC1) were located towards the positive end of the first principal axis while the negative end represented less responsive populations (Fig. 2-1). The most pronounced separation along this sensitivity vector was between Asian ecotypes, which could consistently be found in the negative, UV-B-tolerant part of the vector, and several common cultivars towards the UV-B-sensitive, positive end of the axis, with 'Kopu' and 'Huia' at the top. Within the cultivars, the populations 'Prestige' and 'Sustain' were placed towards the central and more negative region of PC1 (Fig. 2-1). Low latitude ecotypes tended to distribute around the central area of the axis and there was no clear demarcation to the high latitude ecotypes from Scandinavia. *T. repens* populations with

conspicuous differences in pigmentation were heterogeneous in their UV-B response (Fig. 2-1). Pronounced UV-B tolerance could be observed for 'Luclair', intermediate sensitivity for 'Green' and 'Feathermark', and relatively high UV-B sensitivity for 'Lime'.

Plant trait responses in the second principal component

The second principal component (PC2) accounted for 16% of the variance in the mean differences. The trait combination determining the overall UV-B response in PC2 was largely composed of the remaining traits which did not correlate with PC1, namely effects on plant dry matter allocation and epidermal characteristics (Table 2-3). There were eight correlations, most of which could be found towards the positive end of the axis (Fig. 2-1). This end represented higher UV-B sensitivity mainly due to combined effects on below-ground parameters, SLM and stomatal distribution. The most pronounced feature of UV-B tolerance (high negative PC2 scores) was preferential biomass allocation towards growth of roots, rather than shoots (higher RSR), concomitant with a decrease in relative leaf mass (lower LWR). Consequently, UV-B sensitivity in PC2 (high positive PC2 scores) was characterised by no preferential pattern of biomass allocation towards root growth and accompanied by larger absolute decreases in root biomass (Fig. 2-1). Other allocation changes linked to PC2 were those that occurred within the *T. repens* leaf. High positive PC2 scores correlated with a relative shift of stomata towards the abaxial leaf surface while high scores at the opposite end of the axis were linked to increases in specific leaf mass. This was also associated with increases in adaxial stomatal density, paired with UV-B-induced reductions in adaxial epidermal cell size. Finally, there was a marginal relationship of PC2 with changes in petiole length. Change in intrinsic chlorophyll fluorescence yield was the only plant attribute that showed no association with either multivariate sensitivity axis (Table 2-3).

Population responses in the second principal component

A characteristic feature in the second PCA dimension was that several populations which were neutral in PC1 appeared towards the positive (UV-B-sensitive) end of the axis in PC2, including ecotypes from Italy and New Zealand and the breeding lines 'Feathermark' and 'Green' (Fig. 2-1). Populations less responsive to UV-B based on the

PC2 trait combination included 'Sustain', 'Sarikamis', 'Tienshan' and the two Swedish ecotypes.

The focus of this paper is on the first two dimensions as no new plant attributes were linked to the subsequent lower-ranked PC3 and PC4 dimensions, showing fewer and lower correlations for changes in plant attributes and no mechanisms of UV-B sensitivity for populations which displayed UV-B-tolerance in the earlier dimensions (data not shown). Moreover, intrinsic yield of chlorophyll fluorescence (F_v/F_m) continued to make no contribution to the characterisation of the UV-B response with a low cumulative squared correlation coefficient (R^2) of 0.10, even after four principal components.

Correlation analyses

A further aim was to examine possible links between plant characteristics of the *T. repens* populations and PC1, the dimension identified by PCA as the global (overall) measure of their UV-B-responsiveness. Regression analyses revealed that UV-B tolerance (negative PC1 score) was related to slow intrinsic growth rate (Fig. 2-2a) and small constitutive epidermal cell size (Fig. 2-2b). Rather than linked to constitutive UV-absorbing compound levels (Fig. 2-2c), UV-B tolerance was correlated to accumulation of these compounds under UV-B (Fig. 2-2d).

Additional correlations related to the PCA results revealed that fast-growing *T. repens* populations in general had constitutively larger adaxial epidermal cells ($r = 0.473$, $P < 0.05$) and that in turn large-celled populations had larger leaves ($r = 0.660$, $P < 0.001$). Furthermore, the most productive populations displayed lower relative root proportions (low RSR, $r = -0.483$, $P < 0.05$), concomitant with higher relative leaf production (high LWR, $r = 0.583$, $P < 0.01$).

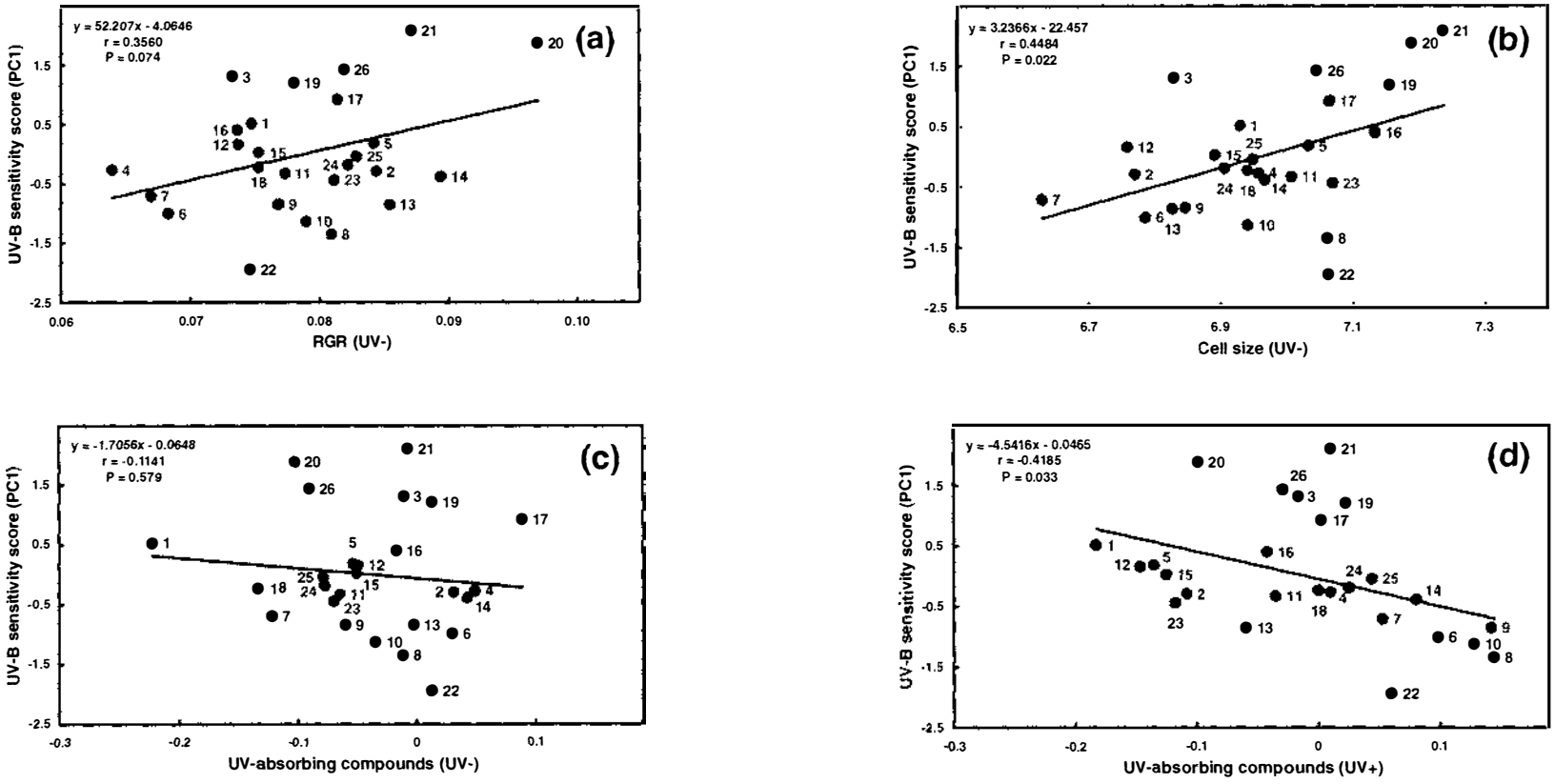


Fig. 2-2. Relationship between overall UV-B sensitivity (PC1 scores) and plant attributes (log-transformed means from PCA) of 26 *T. repens* populations. Numbers identifying the individual populations are given in Table 2-1. PC1 relationship to (a) constitutive (i.e. measured under no UV-B supplementation) relative growth rates; (b) constitutive adaxial epidermal cell sizes; (c) constitutive levels of UV-absorbing compounds; and (d) UV-B-induced levels of UV-absorbing compounds.

Discussion

This study provides for the first time an overall summary of various population responses to UV-B for a plant species based on multivariate analysis of numerous plant attributes. PCA proved useful in exploring the main response patterns among the 26 populations in the 17-dimensional structure. In univariate analyses, *T. repens* was significantly affected by UV-B in most traits measured here (Table 2-2). Whole plant growth characteristics and young leaf growth displayed the strongest decline, while allocation parameters and physiological responses showed more subtle responses.

Plant trait responses in the first principal component

PC1, the most important dimension in describing the overall UV-B response, contained all whole plant growth responses, indicating the integrating nature of these traits as an overall summary for the various individual effects caused by UV-B (Fig. 2-1). Reduced total plant growth is considered the end point of UV-B damage (Stapleton *et al.*, 1997) and the present findings are consistent with other reports showing that growth reduction is due to simultaneous decreases in aerial as well as in below-ground biomass (Krizek *et al.*, 1998; Krizek *et al.*, 1997b). This association also reflects the usual relationship between leaf and root growth (Grime *et al.*, 1997).

Decreases in whole plant growth were associated with reductions in attributes linked to cell division (number of growing points) as well as cell expansion (adaxial epidermal cell size), pointing at both growth-conferring mechanisms underlying the PC1 response. The overall decline in leaf size by 27% (Table 2-2) was accompanied by reduced cell expansion (-10%, Table 2-2) (Fig. 2-1). Thus the remaining portion of leaf size reduction was due to a UV-B-induced decrease in cell division, with epidermal and stomatal cell numbers per leaf (cell density multiplied by leaf size) reduced by 17%. This could suggest a larger contribution of cell division than of cell expansion for the UV-B-induced reduction of leaf size. Similar conclusions have been drawn in pea, with UV-B-generated reductions of 13% for epidermal cell size and of 26% for epidermal cell number contributing to a 39% decrease in leaf size (Nogues *et al.*, 1998). Lower UV-B-induced abaxial cell density, indicating larger cell size, was observed for *Vicia faba* (Visser *et al.*, 1997a) and stimulation of cell division as a consequence of UV-B

irradiation was found in *Petunia hybrida* (Staxen and Bornman, 1994). In conjunction with the current findings this indicates interspecific specificity in the meristematic UV-B response.

The marginal association of PC1 with changes in the concentration of UV-absorbing compounds (Table 2-3) suggests that biochemical deficiencies (no increase in UV-absorbing compound levels) could also bring about high PC1 scores (i.e. pronounced growth reductions). This is consistent with other reports of a relationship between UV-B sensitivity and changes in UV-absorbing compound accumulation (Day and Demchik, 1996; Murali and Teramura, 1986c). UV-absorbing compounds usually include UV-B-screening pigments such as flavonoids and hydroxycinnamic acids (Lavola *et al.*, 1997) and could therefore perform a variety of UV-B-protective functions, including screening and antioxidant activities.

Population responses in the first principal component

PC1 scores for the Asian ecotypes were consistently higher compared to most other populations used in this study, including all *T. repens* cultivars and temperate ecotypes from other geographical regions (Fig. 2-1). Compared to the latter, climatic conditions for the Asian ecotypes are much more continental and the growing conditions generally unproductive (Caradus and Forde, 1996). Their usually small-leafed, low productive phenotype is typical of *T. repens* populations originating in harsh environments (Williams, 1987a). Several of the Asian ecotypes originate from habitats exposed to one or more stress form, including low fertility, low temperature, high altitude and precipitation levels which would be considered marginal for *T. repens* growth (Jones and Lowe, 1993). Predisposition to other forms of stress contributing towards adaptation to enhanced UV-B has been suggested (Al-Oudat *et al.*, 1998) and would be supported by ecological theory on stress tolerance (Diaz *et al.*, 1999; Grime, 1979; Poorter and Garnier, 1999). According to such models of differential plant strategies, populations from stress-prone habitats ('stress tolerators') would more likely experience less severe relative biomass reduction under stress than 'actively foraging' populations from more productive backgrounds ('competitors'). This notion finds additional support from the correlation studies showing lower relative investment into leaf biomass (high LWR) and higher RSR in less productive populations, which would again characterise

them as 'stress-tolerators'. In other studies the Asian ecotypes also were distinct from *T. repens* cultivars such as 'Huia' on the basis of lower productivity, smaller leaves and lower cyanogenesis (Caradus and Forde, 1996).

A link to ecological specialisation is further supported by the observation that many of the more productive *T. repens* cultivars (Table 2-1b) can be found towards the UV-B-sensitive region of PC1. The pronounced UV-B sensitivity in PC1 for 'Huia' is less expected as this general-purpose cultivar has displayed a wide range of adaptability to various environments (Caradus and Woodfield, 1997). Most of the cultivars however are bred for high productivity and it is possible that such specialisation places them at a disadvantage for adaptation to UV-B. The positioning of 'Prestige' and 'Sustain' towards the central and slightly negative, more tolerant part of the sensitivity vector distinguished them from the other cultivars (Fig. 2-1). 'Prestige' has displayed relative tolerance to other forms of stress (e.g. drought) and grows in regions in northern New Zealand with higher background UV-B (Table 2-1b). Relative UV-B adaptability for 'Sustain' might also be related to plasticity resulting from a diverse genetic background including New Zealand, US and Mediterranean germplasm.

From the distribution of Scandinavian and tropical ecotypes it appears that there is no general distinction in UV-B-sensitivity based on difference in latitudinal origin (Fig. 2-1). UV-B susceptibility in plants from higher latitudes has been reported (Barnes *et al.*, 1987). Other studies, however, point at lesser importance for latitudinal origin in explaining differential UV-B adaptation (Robakowski and Laitat, 1999). There was also no relationship between latitudinal origin and UV-B sensitivity in 188 rice cultivars (Dai *et al.*, 1994). The difference in UV-B sensitivity between Scandinavian and Asian ecotypes could suggest that latitudinal differences might still play a role among populations sharing similar features of plant and habitat productivity. Furthermore, the Asian *T. repens* populations are considered to be particularly adapted to extreme changes in environmental conditions (Forde, 1989) and the possibility cannot be excluded that adaptation for the Swedish ecotypes would have required more extended periods of UV-B exposure.

Heterogeneity in the UV-B response was apparent for *T. repens* populations displaying prominent differences in leaf pigmentation (Fig. 2-1). Lower constitutive chlorophyll

levels in the breeding line 'Lime' did not confer additional UV-B sensitivity compared to the mother population 'Huia'. 'Luclair' and 'Feathermark' are known to contain higher anthocyanin levels (Table 2-1b). In particular, high anthocyanin levels have been reported in the leaf mark region for 'Luclair' (Lenoble and Papineau, 1970) and other reports suggest a relationship between higher anthocyanin accumulation and UV-B tolerance (Burger and Edwards, 1996; Stapleton and Walbot, 1994). A recent review points at several possible mechanisms of protective anthocyanin function, including antioxidant activities (Chalker-Scott, 1999). Moreover, it could be argued that UV-B is filtered in the clear portions of the 'Luclair' lamina, which in addition to anthocyanins contains UV-absorbing compounds, while photosynthetically active radiation would be transmitted at least to some degree in these regions due to the lack of chlorophyll. This would effectively provide a UV-B filter for canopy leaves growing underneath such translucent regions. Furthermore, cell shape can be expected to be altered to a large degree in extended leaf mark regions such as in 'Luclair' (Williams, 1987b), which could also lead to differences in dispersion of UV-B within the leaf.

Taken together, the first principal component suggests a UV-B-induced separation among the *T. repens* populations that can largely be explained by ecological specialisation. In particular, morphological stress response and deficiencies in biochemical adaptation appear more pronounced in UV-B-sensitive 'competitor' - rather than UV-B-resistant 'stress-tolerator' populations.

Plant trait responses in the second principal component

Rather than representing decreases in morphological and growth attributes to UV-B (as in PC1), PC2 indicated several UV-B-induced morphogenetic shifts between plant characteristics. Thus this vector appears to constitute a more subtle tier of UV-B responsiveness.

A predominant feature in PC2 was association with changes in aerial and below-ground biomass allocation (Table 2-3). A relative percentage increase of root mass leading to a higher root:shoot ratio under UV-B could suggest an adaptive mechanism redistributing carbon towards plant parts not exposed to UV-B. While some studies report decreases or no changes in the root:shoot ratio under UV-B (Gwynn-Jones and Johanson, 1996;

Sullivan and Teramura, 1988), other investigators also observed increases in relative biomass allocation towards roots in response to UV-B (Barnes *et al.*, 1993; Tosserams *et al.*, 1996). UV-B sensitivity in PC2 was also characterised by smaller increases in leaf dry mass production, relative to leaf size (specific leaf mass, SLM) (Table 2-3). Increased SLM can be due to either increased leaf density or thicker leaves and has been interpreted as an adaptive feature in response to UV-B, changing absorption properties of the leaf (Britz and Adamse, 1994).

Further responses associated with high PC2 scores included changes in epidermal characteristics (Table 2-3). Increases in stomatal density were most likely due to the simultaneously occurring decreases in epidermal cell size, thus leading to a closer, more compact arrangement of the stomata. Stomatal density has displayed a diversity of responses to UV-B, including decreases (Dai *et al.*, 1995; Tevini *et al.*, 1983b), no change (Staxen and Bornman, 1994; Visser *et al.*, 1997a) and increases (Stewart and Hoddinott, 1993). A relative shift of stomata towards the abaxial leaf surface at high positive PC2 scores could indicate a morphogenetic response that differentiates stomata away from direct UV-B exposure. Such a direct effect of UV-B on stomata is suggested in rice, showing greater loss in stomatal density from the adaxial, rather than abaxial surface (Dai *et al.*, 1995). However, decreased stomatal numbers due to UV-B have been observed for the abaxial, but not the adaxial, leaf surface in *Vicia faba* (Visser *et al.*, 1997a).

It was of additional interest to note that intrinsic efficiency of PS II (F_v/F_m) showed no contribution to the overall UV-B responses represented by the main principal components. This is supported by recently reviewed evidence questioning the role of intrinsic PS II efficiency as a key response of UV-B sensitivity (Allen *et al.*, 1998).

Population responses in the second principal component

Several populations displaying moderate UV-B responsiveness in PC1 appeared to be affected in this second dimension of UV-B sensitivity, including temperate ecotypes from Italy and New Zealand (Fig. 2-1). While not selected for yield, these populations generally derive from relatively productive environments, which could help explain generally higher principal component scores in both dimensions compared to the

continental Asian ecotypes originating from lower productive habitats. Furthermore, while unselected, the two New Zealand ecotypes have their genetic origin in the cultivar 'Huia' (Table 2-1a), which already proved to be UV-B-sensitive in PC1. The PC2 trait combination as a measure of UV-B responsiveness was also found for two other populations of intermediate sensitivity in PC1, namely the pigmented lines 'Feathermark' and 'Green', both also of 'Huia' origin (Table 2-1b). It thus appears that cultivars such as 'Huia' which experience long-term ecotypic adaptation or selection for particular pigmentation (higher chlorophyll or anthocyanin levels) can show less sensitivity in direct UV-B-related growth reductions (PC1) but are still UV-B-responsive in several aspects of biomass allocation (PC2).

Populations on the opposite end of PC2 included 'Sarikamis' and 'Tienshan', which also distributed in the negative, more tolerant part of PC1 (Fig. 2-1). These populations derive from high altitude habitats, with adaptation to a number of stress forms including low fertility, low temperatures and low precipitation (Table 2-1a). Similar to PC1, 'Sustain' and – to a lesser degree – 'Prestige' again performed best among the cultivars. In addition, the two Swedish ecotypes also appeared in this spectrum, suggesting lesser overall importance of the PC2 trait combination for the UV-response in these populations.

PC2 thus offers possible explanations for the nature of UV-B sensitivity in populations of intermediate overall (PC1) UV-B responsiveness, largely by affecting allocation attributes between and within plant organs. Moreover, it further points at particularly UV-B-tolerant populations, which not only do relatively well on the main separation axis (PC1), but also rank highly in aspects of the next important dimension ('Sarikamis' and 'Tienshan').

Relationship between PC1 and plant features

From the regression studies it appears that *T. repens* populations which are fast-growing (Fig. 2-2a) as well as those with larger adaxial epidermal cells (Fig. 2-2b) are more sensitive in their overall UV-B response. In addition, while constitutive UV-absorbing compound levels showed no relationship to PC1 (Fig. 2-2c), populations with the capacity to accumulate higher levels of these compounds under UV-B were less

sensitive in the first principal dimension (Fig. 2-2d). Thus in addition to the results from PC1 showing a relationship to UV-B responsiveness of *relative changes* (i.e. treatment values compared to control values) in these plant attributes, the overall reaction to UV-B also appears to be linked to *absolute values* in these features. Absolute, rather than relative accumulation of UV-absorbing compounds has also been linked to UV-B protection, for instance in rice (Sato and Kumagai, 1997). The positive relationship between UV-B sensitivity and high constitutive growth rate is further supported by ecological theory of stress tolerance, suggesting higher stress susceptibility for populations investing in high dry matter production (Diaz *et al.*, 1999; Grime, 1979).

The link between larger constitutive epidermal cell size and UV-B-sensitivity could also be related to plant productivity. The correlation studies show that the fast-growing populations generally had larger cells and that large-celled populations also had larger leaves. Large epidermal cells could therefore also be seen as providing a morphological basis for enhanced resource acquisition and - as outlined above - this is linked to UV-B sensitivity. From a plant strategy perspective (Grime, 1979; Poorter and Garnier, 1999) it is also possible that populations developing smaller epidermal cells in response to environmental stress could have maintained - or even increased in compensation - the capacity to produce compounds of relevance for stress protection. Higher cellular concentrations of epidermis-specific pigments such as flavonoids would contribute to UV-B protection and other experiments demonstrated that smaller-celled *T. repens* populations described here (Fig. 2-2b) contained the highest flavonoid levels (Chapter 6). Finally, there could also be differences in leaf optical properties depending on epidermal cell size. Differences in constitutive epidermal cell size and shape have been related to differences in light focussing and scattering (Vogelmann *et al.*, 1996). Microscopy studies conducted on 'Huia' (sensitive in PC1) and 'Tienshan' (tolerant in PC1) revealed that the smaller adaxial epidermis cells in the latter population had a more convex appearance (see Chapter 8, Fig. 8-1).

Conclusions

T. repens displayed a number of morphological and physiological responses to UV-B under controlled environmental conditions. Reduced cell division and cell expansion lead to smaller leaves, fewer stolons and ultimately to decreases in whole plant growth. PCA and other multivariate assessments could provide useful tools for future examinations of UV-B-induced intraspecific plant responses. The relationship of UV-B sensitivity to habitat productivity and in turn to aspects of plant productivity and physiology (relative growth rate, epidermal cell size, accumulation of UV-absorbing compounds) suggests the existence of plant functional types within *T. repens*. Summary representation of the morpho-physiological responses in PCA indicates higher sensitivity to UV-B for productive populations, mainly reflected in decreases in plant growth. Thus trade-offs appear to exist between morphological and physiological plant features with strongest UV-B-induced relative biomass decreases in populations that invest most metabolic energy in gaining that biomass.

Chapter 3. Growth responses to ultraviolet-B radiation in *Trifolium repens* populations depend on water availability, productivity, and duration of stress

White clover growth under controlled environmental conditions

This chapter has been submitted to *New Phytologist*.

Abstract

This study examined how drought stress might modify the response of white clover to ultraviolet-B radiation (UV-B). Nine white clover populations from a variety of different habitats were used to investigate intraspecific differences in the stress response. Several harvests were used to examine the effect of time. Plants were grown under controlled environmental conditions for 12 weeks with and without supplemental application of 13.3 kJ m^{-2} d^{-1} UV-B and drought was applied during the last four weeks of the experiment. At the end of the experimental period, white clover growth was decreased by about 20% in response to UV-B under well-watered conditions. In drought conditions however, growth reductions due to UV-B were less than half as pronounced, indicating a mitigating effect of moisture deficit for UV-B sensitivity. The extent of the UV-B effect differed among white clover populations under well-watered conditions, with greatest UV-B tolerance observed in slow-growing ecotypes characterised by low original habitat productivity. White clover cultivars selected and bred for yield and agricultural performance displayed higher sensitivity to UV-B. However, drought appeared to increase UV-B tolerance for these cultivars as no pronounced population differences in UV-B responsiveness could be detected under limited water supply. Analysis of the time course of UV-B responsiveness revealed that UV-B sensitivity decreased both with increasing exposure to drought and with longer duration of UV-B irradiation. In conclusion, UV-B effects in white clover can be modified by drought and the extent of this depends on the duration of stress. Furthermore, slower growing populations from environments exposed to multiple stresses are likely to display higher UV-B tolerance.

Introduction

Stratospheric ozone loss leads to increased summertime ultraviolet-B radiation (UV-B, 290-315 nm) (McKenzie *et al.*, 1999). This highlights the need for examinations of UV-B responses in conjunction with other forms of stress that can simultaneously affect plant growth during high UV-B episodes. White clover plants in New Zealand pasture ecosystems often experience pronounced periods of drought during summertime when UV-B levels are highest (Campbell *et al.*, 1999). Drought is one of the most limiting environmental factors to plant productivity, for instance by decreasing plant growth rates, leaf expansion, stem elongation and stomatal conductance (Daie, 1988; Hsaio,

1973). Similar plant responses have been observed under elevated UV-B radiation and numerous findings also suggest reduced plant productivity as a possible result of elevated UV-B irradiation (Drilias *et al.*, 1997; Murali and Teramura, 1986a; Nogues *et al.*, 1998; Sullivan and Teramura, 1990).

Over the last two decades more than 20 studies have investigated the combined effects of UV-B and drought on plant attributes. While such changes can be additive (Teramura *et al.*, 1984b), several reports show modifications of the UV-B effect either by antagonistic or by synergistic interactions. The latter response, represented by enhanced UV-B sensitivity under drought has been observed for growth in the moss *Hylocomium splendens* (Björn *et al.*, 1997), the shrub *Nerium oleander* (Drilias *et al.*, 1997) and radish (Tevini *et al.*, 1983a). Frequently however, ameliorating antagonistic effects of drought for UV-B sensitivity have also been reported, e.g. for soybeans (Murali and Teramura, 1986a; Sullivan and Teramura, 1990; Teramura *et al.*, 1990), *Pinus* (Manetas *et al.*, 1997; Petropoulou *et al.*, 1995) and cowpea (Balakumar *et al.*, 1993).

Several studies have revealed between-species differences in plant responses to the combined application of UV-B and drought, for example in *Brassica* (Conner and Zangori, 1998), *Pinus* (Petropoulou *et al.*, 1995), cucumber and radish (Teramura *et al.*, 1983; Tevini *et al.*, 1983a), wheat and wild oat (Barnes *et al.*, 1995) and European heathland species (Björn *et al.*, 1997). There are also indications of within-species differences for UV-B responses in combination with drought from comparisons of two soybean cultivars in the field (Murali and Teramura, 1986b; Teramura *et al.*, 1990). A number of other reports have shown that within-species differences can influence plant growth responses to UV-B alone (Barnes *et al.*, 1993; Biggs *et al.*, 1981; Correia *et al.*, 1998; Dai *et al.*, 1994; Murali and Teramura, 1986c; Pukacki and Modrzynski, 1998; Teramura *et al.*, 1990). Furthermore, there are also indications of population differences for drought tolerance in white clover (Woodfield and Caradus, 1987).

It has been suggested that adaptation to one stress can facilitate tolerance to other forms of stress (Grime, 1979; Manetas *et al.*, 1997; Petropoulou *et al.*, 1995). Some studies also indicate UV-B tolerance for plants originating from habitats of higher ambient UV-B levels (Barnes *et al.*, 1987; Pukacki and Modrzynski, 1998; Sullivan *et al.*, 1992) as well as for drought-tolerant species (Al-Oudat *et al.*, 1998). There is a need for more

comprehensive investigations on possible linkages between UV-B responsiveness and adaptation to environmental conditions other than background UV-B levels. This warrants inclusion of a variety of populations of the same species. Population comparisons strongly facilitate comparability between plant types because they are not affected by species-specific differences.

The majority of studies investigating the combined effects of UV-B and drought have been conducted in the field. Such studies were often based on altering water supply due to seasonal differences in precipitation, but only a few specifically included differential water treatments. While field-based research provides natural environmental conditions (e.g. temperature, humidity and herbivory), plant responses to these conditions can potentially mask or interact with effects caused by drought and by UV-B (Allen *et al.*, 1999; Teramura *et al.*, 1990). Investigations of UV-B responses in combination with drought under controlled environmental conditions allow direct investigation of processes inherent to this important stress interaction without other confounding environmental influences.

Nine white clover populations from a wide variety of environmental backgrounds were exposed to a combination of 12 weeks elevated UV-B and four weeks drought under controlled environmental conditions. The objective was to determine overall UV-B sensitivity for white clover, based on measurements of aboveground biomass accumulation as a measure of plant productivity. It was hypothesised that this response would be modified by water stress and differ among white clover populations. A further aim was to examine possible relationships of UV-B sensitivity with the habitat and productivity of the white clover populations. Additionally, several harvests were used to examine whether UV-B responsiveness would change over time.

Materials and Methods

Experimental design

A 2 x 2 x 9 factorial design included two levels of UV-B radiation, two watering regimes and nine populations of white clover. There were five replicated pots per population, each pot containing 10 plants. To minimise effects from differential shading under the UV-B rigs, pots were arranged randomly on six trolleys and the trolleys were rotated daily in the two rooms using a standardised pattern allowing different positions for 24 consecutive days before the starting position was resumed. Plant biomass in droughted plants was harvested the day after rewatering in order to prevent collection of wilted material.

Plant cultivation and UV-B irradiation

Seeds of nine different white clover populations were obtained from the Margot Forde Germplasm Centre in Palmerston North, New Zealand. These populations were chosen to provide a range of plant types from differential environmental backgrounds (Table 3-1). The study included three slow-growing ecotypes collected in the wild from habitats exposed to multiple stresses ('Sarikamis', 'Tienshan' and 'Häggås'), four cultivars multiplied in breeding programmes for agricultural yield ('Huia', 'Kopu', 'Haifa' and 'Prestige'), and two breeding lines (un-multiplied selections). The latter included the high-yielding population 'Octoploid' and the less productive 'Syrian', selected for tolerance to leaf rust and drought (Table 3-1). Following scarification, seeds were placed in petri dishes on moist filter paper and incubated at 25°C for 3-5 days.

Table 3-1. Description of *Trifolium repens* ecotypes (populations collected in the wild), cultivars (populations multiplied for high agronomic value) and breeding lines (un-multiplied selections). Accession numbers are from the Margot Forde Germplasm Centre in Palmerston North, New Zealand. References for ecotypes: 'Häggås' (Nordiska Genbanken, 1993), 'Sarikamis' (Caradus and Forde, 1996), 'Tienshan' (Forde, 1987). References for cultivars from Caradus (1986) and Caradus and Woodfield (1997), and for breeding lines: 'Syrian' (Caradus *et al.*, 1990a), 'Octoploid' (Woodfield and Cousins, 1999).

Accession	Type	Name	Country	Description
C15130	Ecotype	Häggås	Sweden	Latitude 64.2°N, 500m altitude, 700mm annual rainfall, south-west clay slope, abandoned cultivation.
C8734	Ecotype	Sarikamis	Turkey	Latitude 40.2°N, 2300m altitude, 580mm annual rainfall, basaltic, extended snow cover and clouds, very cold.
C8749	Ecotype	Tienshan	China	Latitude 43.0°N, 2000m altitude, 600mm annual rainfall, hay meadow, heavy winter snow, very cold.
C13173	Cultivar	Haifa	Israel/ Australia	Bred from Israel germplasm originating near 33°N latitude, high yielding, heat- and salt-tolerant, more sensitive to frost and drought, decreased persistence under grazing, relatively poor disease resistance, good performance in lowland conditions.
C7544	Cultivar	Huia	New Zealand	Long-established New Zealand general purpose cultivar, selected for yield and persistence to grazing, high phenotypic plasticity, high amino acid content, sensitive to P-deficiency, water stress and frost, relatively poor disease resistance, best suited for lowland conditions.
C13176	Cultivar	Kopu	New Zealand	Includes germplasm from ladino cultivars, cv. 'Pitau' and a Spanish strain, bred for lowland farming, nematode resistance and yield, more productive and leaves more upright habit than 'Huia', recommended for grazing, large roots but no persistence in dry hill country, frost sensitive.
C11678	Cultivar	Prestige	New Zealand	Bred from ecotypes in Northland (northern New Zealand), improved heat and drought tolerance, grazing-tolerant, suited for hill country climate and dry sunny slopes, relatively good disease resistance.
C18791	Breeding line	Octoploid	New Zealand	Higher ploidy level (8x), parentage from cultivar 'Dusi' with tall, large-leafed productive phenotype, large root system with persistence in soils with drought incidence, more frost-sensitive than 'Huia'.
C10575	Breeding line	Syrian	Syria	Selected from Syrian germplasm for disease tolerance (leaf rust), improved persistence in drought conditions under grazing.

The emerging seedlings were subsequently transplanted into square gallon pots containing a sterilised sand/mineral mix and transferred into large growth chambers at the National Climate Laboratory in Palmerston North. The pots were supplied with an automatic drip irrigation system with half-strength Hoagland's nutrient solution at regular intervals during the 12 h day and 12 h night cycle and flushed with water weekly. The experiment was conducted for 12 weeks in growth rooms of identical design and under continuous monitoring of environmental conditions. In other studies these rooms demonstrated excellent environmental control with insignificant differences between rooms (Warrington *et al.*, 1999; Warrington and Kanemasu, 1983). The average photosynthetic photon flux (PPF) during the light period was 425 µmol m^{-2} s^{-1}, supplied by a water-screened array of four 1 kW Sylvania 'metal-arc' high pressure discharge lamps, together with four 1 kW Philips tungsten iodide lamps. PPF was measured using a LI-COR 185 meter with a LI-190S quantum sensor. Temperatures were 22°C during the day and 18°C at night, with the respective mean CO_2 levels at 367ppm and 383ppm. Relative humidity was 70-80%.

One hour after onset of the light period until one hour before darkness, UV-B radiation was supplied by Philips TL 40W/12 RS UV fluorescent tubes, enclosed in cellulose diacetate filters. The UV-B irradiance levels were gradually increased during the first three weeks of UV-B supplementation to facilitate acclimation of the plants to UV-B. After this, plant-weighted (Caldwell, 1971) biologically effective UV-B ($UV\text{-}B_{BE}$) levels (normalised to 300 nm) were 13.3 kJ m^{-2} d^{-1}, mimicking 25% summer ozone depletion above Palmerston North, New Zealand. The tubes were suspended 500 mm above plant level. A Solar Light Company model 501A UV biometer sensor measured the UV output which was controlled by a feedback system to maintain the constant, pre-set UV-B irradiation doses in response to tube ageing and cellulose diacetate filter degradation. These filters were changed when the feedback system output approached maximum voltage. A Bentham DM150 UV spectroradiometer was used to calibrate the sensor at regular intervals against the generalised plant action spectrum (Caldwell, 1971).

Harvesting of plant material and drought application

During the 12-week experimental period, plants were clipped at four harvests near pot height to collect plant biomass. This also minimised effects of shading and prevented the plants from exceeding pot size. The first harvest was conducted four weeks after establishment. The second harvest was performed three weeks later, followed by the onset of the drought period one week thereafter. A third harvest was conducted midway between this and the fourth harvest which concluded the drought treatment after four weeks. The third harvest included growth in non-drought and developing drought conditions and thus represented a less severe water stress treatment compared to the final harvest.

The following treatments were applied during the four-week drought period:

(1) UV- W+ No UV-B supplementation and well-watered conditions.

(2) UV+ W+ UV-B supplementation and well-watered conditions.

(3) UV- W- No UV-B supplementation and water limitation.

(4) UV+ W- UV-B supplementation and water limitation.

Drought treatment was applied by withholding water and monitored gravimetrically using techniques employed in other studies of water stress in white clover (Barbour, 1996; Barbour *et al.*, 1996). Plants in droughted pots were rehydrated when half or more of their petioles showed signs of incipient wilting. At this point each pot was weighed and watered to 4% soil moisture content, with no loss from drainage after rewatering. In the well-watered treatment, the normal watering and nutrient solution regime was continued.

Aerial biomass from each harvest was dried for 48 h at 80°C and weighed. The accumulated biomass from these harvests was used to calculate the rate of dry matter production. Sensitivity to UV-B was expressed as the percentage change calculated from the comparison of dry matter production between UV-B-treated and control plants.

Statistical analysis

The General Analysis of Variance procedure in GENSTAT (Genstat, 1993) was used as the principal statistical method for analysis of main and interaction effects on aerial biomass accumulation transformed to a logarithmic scale. Repeated measurements of biomass collected in four harvests allowed testing for the effect of time on main and interaction terms. This was examined with the method of residual maximum likelihood (REML) in GENSTAT (Genstat, 1993). The Arepmeasures procedure (ANOVA for repeated measurements) was used to characterise the type of correlation between observations of the four harvests (Genstat, 1993). The resulting correlation matrix revealed a typical auto-regressive structure (AR1) for the covariance model and the REML analysis was specified accordingly. For conciseness, the overall REML analysis incorporating all treatments is presented here. Individual REML analyses distinguishing between droughted and well-watered samples gave similar results. The relationship between UV-B sensitivity and constitutive productivity (i.e. biomass production without UV-B supplementation) of the white clover populations was examined with the GENSTAT Regression Analysis procedure.

Results

Influence of drought on UV-B sensitivity

At the end of the experiment (final harvest, Fig. 3-1a), UV-B significantly decreased biomass accumulation by about 20% across white clover populations in well-watered conditions, compared to a marginal UV-B-induced reduction by about 8% under moisture deficit. Examination of the UV-B × drought interaction at final harvest showed that this represented a significant mitigating effect of drought for UV-B sensitivity ($P < 0.05$). UV-B-induced growth decreases were almost equal at around 30% under both water regimes following drought establishment (at harvest 3, Fig. 3-1b). Mediation of the UV-B effect depended on the duration of drought (significant harvest × UV-B × drought term in REML analysis of biomass accumulation during drought, Table 3-2).

Table 3-2. Residual maximum likelihood (REML) analysis of log-transformed aerial biomass production of nine white clover populations collected in four harvests during 12 weeks growth under supplementation with or without UV-B of 13.3 kJ m^{-2} d^{-1}. Half of the samples collected during the final two harvests also experienced four weeks of water limitation.

Plant Response	*P*
Harvest	< 0.001
UV-B	< 0.001
Water	< 0.001
Population	< 0.001
Harvest × UV-B	< 0.001
Harvest × Water	< 0.001
UV-B × Water	0.584
Harvest × Population	< 0.001
UV-B × Population	0.011
Water × Population	0.033
Harvest × UV-B × Water	0.003
Harvest × UV-B × Population	0.808
Harvest × Water × Population	< 0.001
UV-B × Water × Population	0.463
Harvest × UV-B × Water × Population	0.935

At final harvest (Fig. 3-1a) the extent of growth reduction after 12 weeks of UV-B irradiation was less than half that of four weeks of drought treatment across the white clover populations. At final harvest, the average drought-induced productivity decrease across populations was 71% in non-irradiated plants, compared to a drought-elicited growth reduction of 65% under UV-B (Fig. 3-1a). Examination of the UV-B × drought interaction revealed that this difference in the drought response was non-significant between the two UV-B treatments. Drought effects were even more comparable at the first harvest of the droughted samples (harvest 3), showing near equal growth reductions by about 57% in both UV-B regimes (Fig. 3-1b). A significant harvest × drought term (Table 3-2) revealed that water stress-elicited biomass reduction became greater with increasing duration of drought.

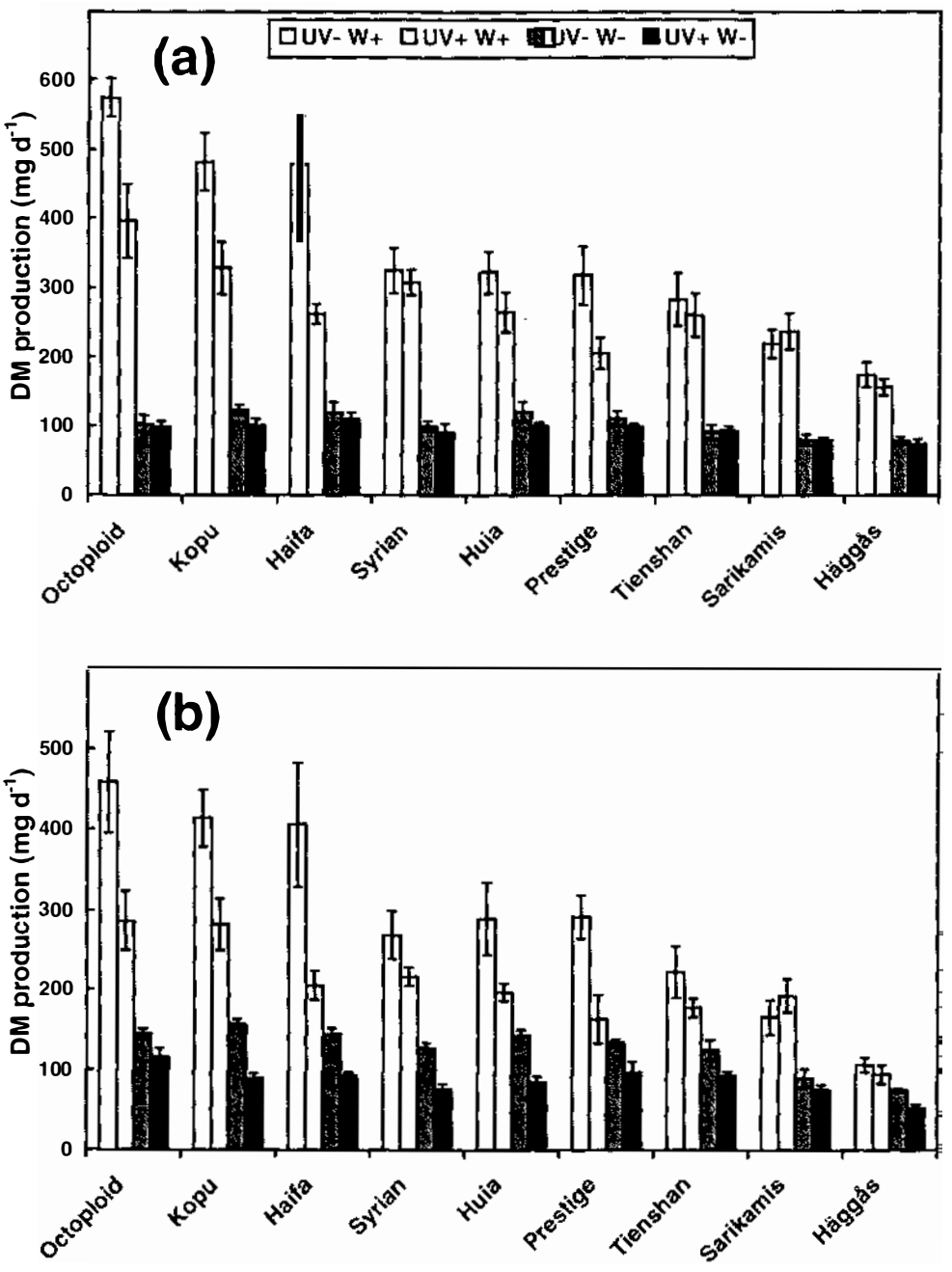


Fig. 3-1. Aboveground biomass production in nine white clover populations grown with (UV+) and without (UV-) supplementation of 13.3 kJ m^{-2} d^{-1} UV-B at (a) final harvest after four weeks drought, compared to (b) the penultimate harvest, 10 days after onset of the drought treatment. The populations are ranked by the productivity of unstressed plants (UV- W+) at final harvest. Error bars are ± SE. W+ = well watered; W- = droughted.

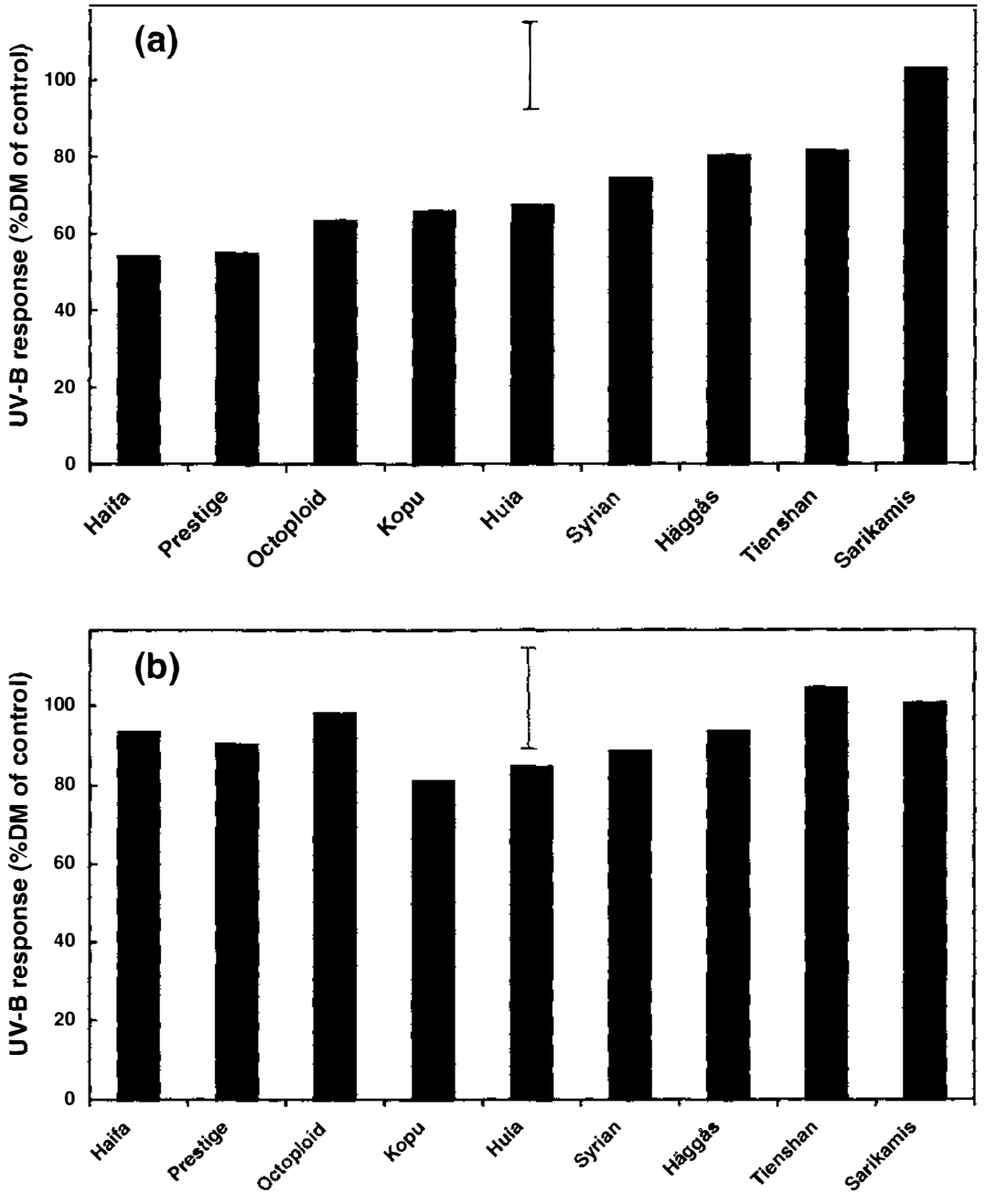


Fig. 3-2. Relative changes in the rate of biomass production for nine white clover populations in response to supplementation of 13.3 kJ m^{-2} d^{-1} UV-B. (a) Accumulated yield of plants grown for 12 weeks under well-watered conditions and (b) plants harvested at the end of the experiment after exposure to drought for four weeks. The populations are ranked by the degree of UV-B sensitivity in (a). Values are the ratios of back-transformed means of logarithm-transformed data. The bars represent the percentage changes required for significant differences ($P < 0.05$) in UV-B sensitivity for the nine white clover populations calculated from the least significant ratio.

Influence of population differences and time on UV-B sensitivity

In addition to significant ($P < 0.001$) within-species differences in constitutive productivity under no UV-B supplementation, there were significant population-specific differences in the degree of the UV-B response in plants growing for 12 weeks under UV-B in well-watered conditions ($P < 0.05$) (Fig. 3-2a). This was linked to the productivity of the white clover populations (Fig. 3-3). Greater UV-B tolerance was found in less productive white clover ecotypes (Table 3-1, Fig. 3-3). 'Sarikamis' performed best among the ecotypes with no UV-B-induced growth reduction, while the more productive white clover cultivars were generally more sensitive to UV-B, with 'Haifa' showing the strongest biomass reduction by more than 40% (Fig. 3-2a). At final harvest the analysis of biomass accumulation under well-watered conditions largely confirmed these population differences in UV-B sensitivity, but productivity of the droughted samples (four weeks of soil moisture deficit) revealed no significant population differences in the UV-B response (Fig. 3-2b).

In addition to an effect of time on UV-B responses under drought, duration of UV-B exposure also affected relative dry matter production under well-watered conditions (Fig. 3-4). On average, the rate of white clover biomass production decreased by 30% during 12 weeks of UV-B exposure under well-watered conditions ($P < 0.001$), but this was more pronounced during initial harvests with a 40% growth reduction, compared to a decrease of about 20% eight weeks later. A significant harvest × UV-B interaction (Table 3-2) underlines that the white clover populations became less UV-B-sensitive with progressing exposure to UV-B. The ranking in UV-B sensitivity among the white clover populations generally remained similar, irrespective of time (non-significant interaction term for harvest × UV-B × population, Table 3-2).

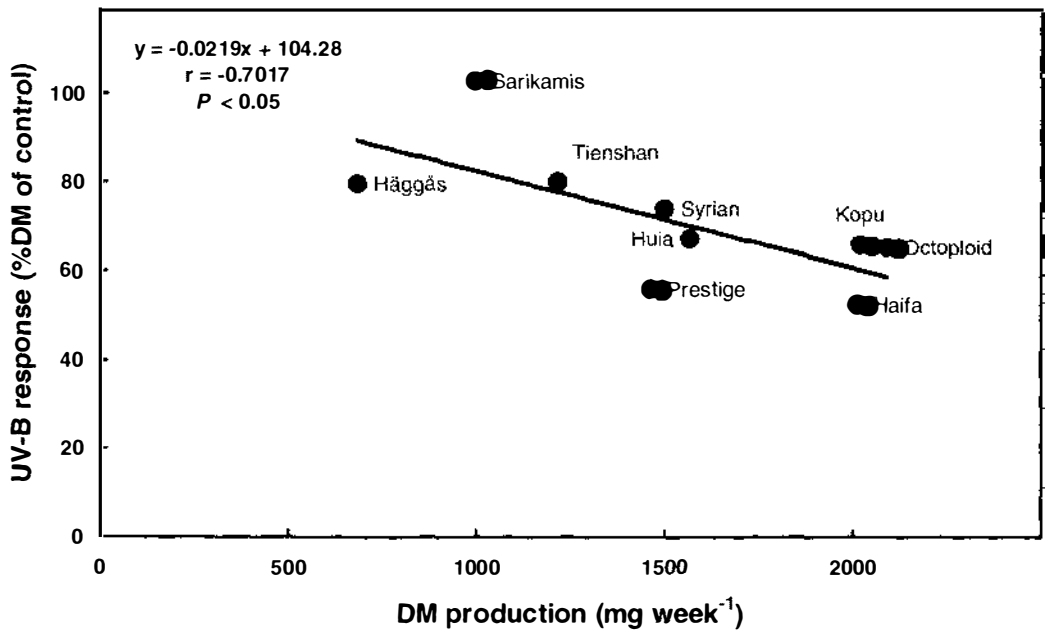


Fig. 3-3. Relationship between constitutive productivity (i.e. biomass production without UV-B supplementation) during 12 weeks growth in controlled environmental conditions and relative growth (dry matter production under UV-B expressed as a percentage of that of the control) in response to elevated UV-B of 13.3 kJ m^{-2} d^{-1} for nine white clover populations. DM = dry matter.

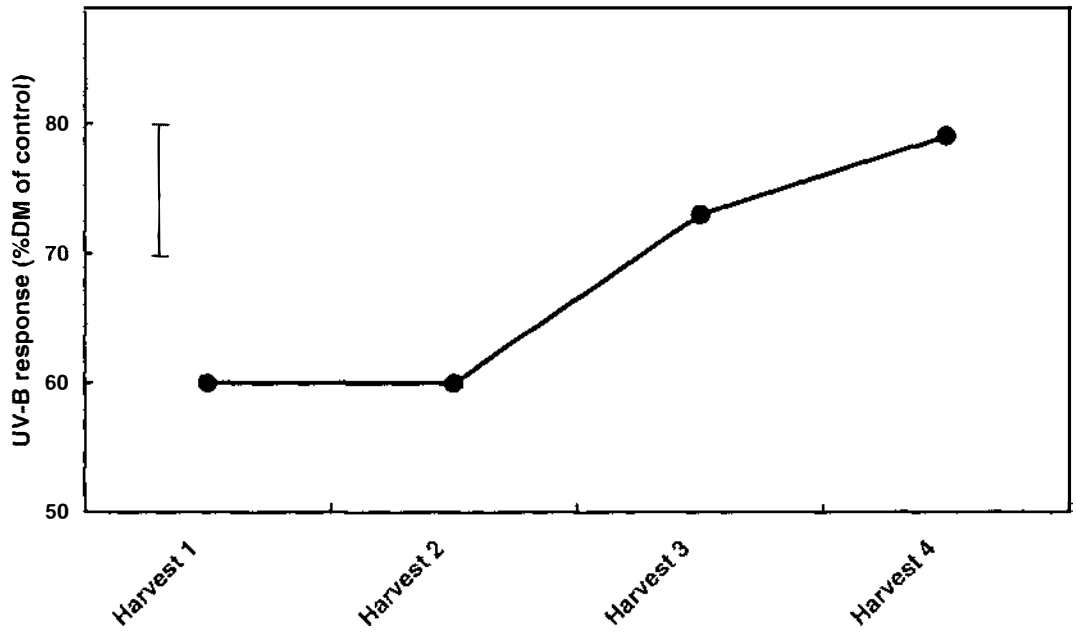


Fig. 3-4. Time course of the mean UV-B response under well-watered conditions measured as relative growth (dry matter production under UV-B expressed as a percentage of that of the control) across nine white clover populations during four consecutive harvests. Plants were grown for 12 weeks with and without supplementation of 13.3 kJ m^{-2} d^{-1} UV-B. The graph shows the ratios of the back-transformed means, constituting an overall summary of UV-B sensitivity for each harvest. The error bar represents the percentage change calculated from the least significant ratio that is required for a significant harvest effect ($P < 0.05$).

Discussion

This study revealed significant reductions in biomass production for white clover after 12 weeks of exposure to UV-B under controlled environmental conditions. Most importantly, it became evident that the main UV-B effect was significantly modified through interactions with drought, white clover population and duration of stress exposure.

Influence of drought on UV-B sensitivity

The results from biomass production after four weeks of drought indicate an antagonistic, ameliorating influence of water stress on UV-B sensitivity in white clover (Fig. 3-1a). Other studies have shown additive (Teramura *et al.*, 1984b), antagonistic (Balakumar *et al.*, 1993; Manetas *et al.*, 1997; Murali and Teramura, 1986a; Petropoulou *et al.*, 1995; Sullivan and Teramura, 1990; Teramura *et al.*, 1990) and synergistic (Björn *et al.*, 1997; Drilias *et al.*, 1997; Tevini *et al.*, 1983a) interactions between UV-B and drought. Studies reporting beneficial effects of drought for UV-B sensitivity have shown that detrimental UV-B effects under well-watered conditions can either be removed (Murali and Teramura, 1986a) or even reversed by drought (Balakumar *et al.*, 1993; Teramura *et al.*, 1990). This has been related to a number of drought-induced morphological and physiological changes of relevance for UV-B protection, including growth delay and radical scavenging (Beggs *et al.*, 1986).

From Fig. 3-1 it appears that with increasing duration and severity of drought, the resulting water deficit assumes the main limiting function for productivity, masking more subtle effects caused by UV-B. Thus the extent of the UV-B × water stress interaction and even its direction can be influenced by the extent of drought. Examination of the UV-B × drought interaction at the first harvest during drought application would have resulted in concluding an additive response, rather than the antagonistic interaction observed at final harvest (Fig. 3-1a,b). Drought alone reduced productivity more than double compared to UV-B. Such pronounced differences in responses to the two stress forms have also been observed in other studies, e.g. in glasshouse studies of *Brassica* (Conner and Zangori, 1998). A masking effect of drought for UV-B sensitivity is in accord with reports from field-grown soybean

(Sullivan and Teramura, 1990), where UV-B effects appeared predominant under well-watered conditions while UV-B-generated growth reductions were obscured when growth was already strongly decreased by drought.

Beneficial interaction between UV-B and drought has also been observed as an improvement of the drought response through preconditioning with UV-B (Nogues *et al.*, 1998). Such effects have been linked to UV-B-induced cuticle thickening, resulting in decreased water loss and improved plant performance under drought (Manetas *et al.*, 1997). In the present study, there was no significant interaction of UV-B with water stress responses, indicating that UV-B did not play a major role in the improvement of drought sensitivity. It cannot be ruled out, however, that such interaction would occur over longer drought periods. In addition, there may be beneficial effects on subsequent recovery from drought stress.

Influence of population differences on UV-B sensitivity

To date, only limited research on UV-B responses in combination with drought has been conducted for populations within the same species. Such intraspecific information is largely based on earlier comparisons between two soybean cultivars, suggesting that population differences in UV-B sensitivity can be altered or removed under drought (Murali and Teramura, 1986b; Teramura *et al.*, 1990). In the present comparison of nine white clover populations, significant differences in the UV-B response were found among white clover populations under well-watered conditions, ranging from no decrease in biomass accumulation in the ecotype 'Sarikamis' to more than 40% growth reduction in the agricultural cultivar 'Haifa' (Fig. 3-2a). However, no significant population differences could be recorded in the UV-B response of droughted plants (Fig. 3-2b). A levelling effect by drought on within-species differences in UV-B responsiveness could therefore be of particular advantage for higher yielding agricultural white clover cultivars.

Population-specific differences in UV-B sensitivity have been demonstrated in a number of plant species, including *Pinus* (Pukacki and Modrzynski, 1998), maize (Correia *et al.*, 1998), rice (Barnes *et al.*, 1993; Dai *et al.*, 1994), cucumber (Murali and Teramura, 1986c) and soybeans (Biggs *et al.*, 1981; Murali and Teramura, 1986b;

Teramura *et al.*, 1990). No UV-B effect on yield was measured within and between a number of forage species under ambient northern hemisphere UV-B levels in the field, including white clover (Papadopoulos *et al.*, 1999). However, that study did not specify the type and environmental background of the cultivars used. A similar study under southern hemisphere UV-B levels showed reduced growth for a New Zealand white clover cultivar (Matthew *et al.*, 1996).

The significant within-species differences for UV-B sensitivity observed here allowed closer examination of possible relationships to the environmental background and productivity of the white clover populations. While some reports suggest a relationship between UV-B responsiveness and natural UV-B backgrounds (Barnes *et al.*, 1987; Pukacki and Modrzynski, 1998; Sullivan *et al.*, 1992), others show no such relationship (Dai *et al.*, 1994; van de Staaij *et al.*, 1995). Comparisons between UV-B-induced growth responses of the white clover populations also suggested no clear relationship to likely UV-B levels at the habitat of origin. The Scandinavian ecotype 'Häggås' (low ambient UV-B) displayed similar UV-B tolerance to the ecotypes from lower latitudes and higher altitudes (Fig. 3-2a).

Nevertheless, another relationship between UV-B responsiveness and habitat of origin became apparent, suggesting a link to habitat productivity. Plant populations exposed to multiple forms of stress in their habitat of origin displayed greater tolerance to UV-B (Table 3-1). Stress factors operating in the natural habitat of the ecotypes 'Sarikamis', 'Tienshan' and 'Häggås' include reduced habitat fertility, cold temperatures and low precipitation (Table 3-1). These factors are all known to elicit defense mechanisms that could also be of potential advantage for UV-B protection, including higher antioxidant levels (Grace *et al.*, 1998; Pizzi and Cameron, 1986). A related study found high accumulation of specific flavonoids and a link to UV-B protection in these ecotypes (see later in Chapter 6, Fig. 6-6). Similarly, the 'Syrian' breeding line derives from drought-prone habitats and was selected for tolerance to leaf rust, a stress form reported to result in higher polyamine (Bharti and Sawhney, 1996) and oxidase activity (Sharma and Sharma, 1997). Thus it appears these other stresses may have resulted in specialisations for these populations that could better adapt them to UV-B.

In addition, a significant relationship was demonstrated between constitutive biomass production and UV-B sensitivity (Fig. 3-3). White clover cultivars selected and bred for agricultural productivity were more affected by UV-B than less productive, unselected ecotypes (Fig. 3-3, Table 3-1). This is in accord with similar findings from large-scale screening of white clover populations using short-term UV-B radiation (Chapter 2). The highly productive breeding line 'Octoploid' contains double the usual tetraploid white clover chromosome set (Woodfield and Cousins, 1999), which could also represent an additional target for UV-B-induced nuclear DNA damage (Campbell and Grime, 1993). Considering relative drought tolerance and origin at latitudes with potentially higher UV-B irradiation (Table 3-1), the observed UV-B sensitivity for 'Prestige' was less expected. However, in other studies 'Prestige' was low in the accumulation of flavonol glycosides of relevance for UV-B protection (Chapter 6). The UV-B-sensitive high-yielding populations 'Haifa' and 'Kopu' have displayed poor performance under a variety of stress forms, for instance in dry conditions and frost (Table 3-1). Similar has also been observed in studies on the general-purpose cultivar 'Huia' (Table 3-1).

Taken together, these results are in accord with ecological models of the evolution of stress tolerance, predicting higher tolerance (less severe relative biomass reduction) for species and populations from low habitat and plant productivity (Grime, 1979; Poorter and Garnier, 1999).

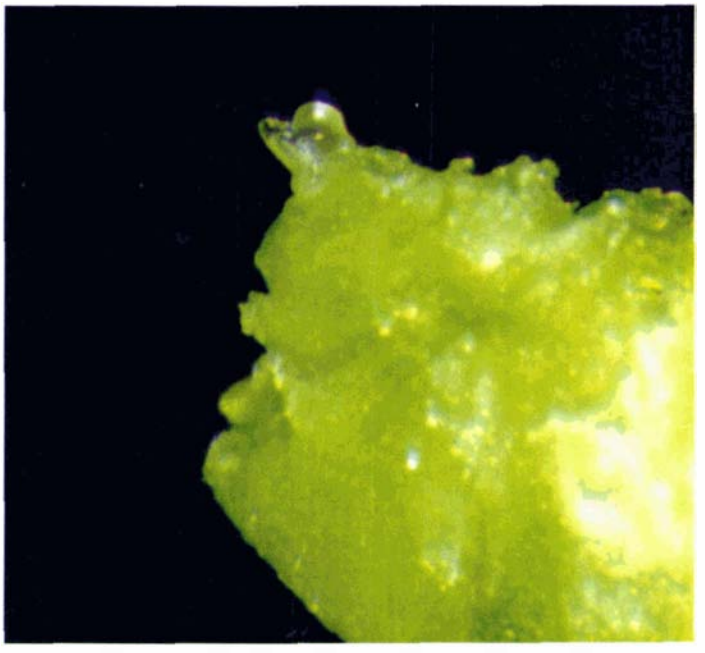
Influence of time on UV-B sensitivity

UV-B sensitivity in white clover also appeared to be influenced by time, suggesting improvements for aerial biomass reductions with increasing duration of UV-B exposure (Fig. 3-4). Some studies report cumulative detrimental effects for plant growth in response to increased duration of UV-B exposure (Björn *et al.*, 1997; Tevini *et al.*, 1983a). Other findings, however, point at beneficial effects of increasing time for UV-B sensitivity. In soybean, growth reductions have been observed in early, but not advanced stages of plant development (Teramura *et al.*, 1984b). In faba beans, decreasing UV-B sensitivity over time has been related to improved photorepair functions, changes in plant architecture and increasing absorbance by UV-absorbing compounds (Visser *et al.*, 1997b). It remains to be tested whether time dependency of

the UV-B response under continuous UV-B exposure would experience beneficial or detrimental modifications during the daily and seasonal UV-B fluctuations in the field.

It is concluded that white clover growth could be less affected by UV-B during periods of drought, compared to moist seasonal conditions. Within-species comparisons show that plant and habitat productivity can serve as predictors for UV-B responsiveness.

Chapter 4. Effects of ultraviolet-B radiation on *Trifolium repens* morphology: mediation by drought and relationships to UV-B sensitivity



Dissected white clover bud with apical dome and leaf primordium

This Chapter has been submitted to *Plant, Cell and Environment.*

Abstract

This study examined effects of UV-B on several aspects of white clover growth and morphology. Comparisons were made with and without drought to determine whether limited moisture availability would alter such responses. Nine white clover populations were examined to investigate possible relationships between morphology and UV-B sensitivity. A factorial design included supplementation with and without 13.3 kJ m^{-2} d^{-1} UV-B for 12 weeks, with and without additional exposure to four weeks of drought. Changes in total biomass production were accompanied by changes in morphological attributes contributing to productivity. In general, UV-B in well-watered conditions reduced leaf mass and leaf size by about 15%, rates of leaf appearance by 5% and stolon elongation by 17%. Under drought conditions, these UV-B-induced changes were only marginal or non-significant. Drought treatment resulted in much greater decreases in these attributes than UV-B. The number of leaf primordia formed in the apical bud was decreased by 4% under UV-B, and by 10% in response to drought. Leaf dry matter allocation attributes (specific leaf mass, SLM and percentage leaf dry mass, PDM) were strongly affected by moisture deficit, rather than by UV-B. Drought increased SLM by 50% and PDM by 40%. UV-B sensitivity in the white clover populations was associated with higher plant productivity, larger leaf size as well as higher PDM under UV-B. Thus morphological attributes conferring fast potential growth under productive conditions carry a cost in the form of higher UV-B sensitivity. The observed amelioration of UV-B sensitivity under drought could be related to pronounced increases in SLM and PDM. These findings propose the existence of distinct functional types within white clover that might be used to predict UV-B sensitivity.

Introduction

Stratospheric ozone loss has been demonstrated to result in increased ultraviolet-B irradiation (UV-B, 290-315 nm) (McKenzie *et al.*, 1999). The temperate pasture legume white clover experiences UV-B levels in New Zealand that are up to 50% higher than at comparable temperate latitudes in the northern hemisphere (Seckmeyer *et al.*, 1995). Several publications have reviewed UV-B effects on agro-ecosystems (e.g. Krupa *et al.*, 1998b; Rozema *et al.*, 1997c), but detailed information on UV-B responses in pasture species is limited, particularly in conjunction with other forms of stress. In the New Zealand pasture ecosystem and many other habitats there is an increased likelihood of

drought coinciding with higher UV-B levels during prolonged clear-sky periods. Drought is one of the most common forms of stress encountered by plants in the field (Hsaio, 1973).

Water availability has been ranked a primary factor impacting on UV-B responsiveness among environmental change processes (Gwynn-Jones *et al.*, 1999a). The combination of UV-B and drought has been investigated in several studies (e.g. reviewed in Bornman and Teramura, 1993; Caldwell *et al.*, 1995). Some examinations point at a synergistic, worsening effect of drought stress for UV-B sensitivity (Conner and Zangori, 1997; Feldheim and Conner, 1996; Gwynn-Jones *et al.*, 1999a) while others found no drought-induced modifications of the UV-B effect (Allen *et al.*, 1999). Reports of additivity for UV-B-induced changes under differential moisture availability also suggest that UV-B responses can act independently of water availability (Conner and Zangori, 1998; Teramura *et al.*, 1984b). Many studies, however, point at ameliorating effects of drought exposure for UV-B responses (Balakumar *et al.*, 1993; Manetas *et al.*, 1997; Murali and Teramura, 1986a; Sullivan and Teramura, 1990; Teramura *et al.*, 1990).

UV-B regulates plant development from molecular to ecosystem levels (Rozema *et al.*, 1997b). It has been suggested that morphological changes are a primary outcome of elevated UV-B radiation (Rozema *et al.*, 1997b). Examinations of internode elongation revealed increases (Björn *et al.*, 1997), no change (Murali and Teramura, 1986a) and decreases in response to UV-B (Gonzalez *et al.*, 1998; Hoffmann, 1999). Leaf area in *Nerium oleander* decreased in response to UV-B under drought, but not in well-watered seasonal conditions (Drilias *et al.*, 1997), while the opposite response has been found for soybean (Murali and Teramura, 1986a; Sullivan and Teramura, 1990). The number of leaves was not affected by UV-B in pea, but showed decreases under drought (Allen *et al.*, 1999; Nogues *et al.*, 1998). Few studies on UV-B-induced leaf area responses have separated effects on leaf size (area per leaf) from those on leaf number (Nogues *et al.*, 1998). Furthermore, little is known about UV-B effects on leaf primordia formation in the apical bud, a process that is affected by other environmental conditions, e.g. temperature and daylength (Thomas, 1987b).

There are also reports on alterations in relative leaf dry matter allocation under UV-B. Findings of UV-B-elicited decreases in fresh mass without simultaneous reductions in dry mass indicate disruptions in plant water relations for a number of plant species, including cucumber, radish, maize and barley (Krizek, 1975; Tevini *et al.*, 1981). Leaf dry mass allocation to each unit of leaf area (specific leaf mass, SLM) has been shown to increase in response to higher UV-B levels (Filella and Penuelas, 1999), in drought conditions (Murali and Teramura, 1986a) or under the combination of UV-B and drought (Balakumar *et al.*, 1993). Drought-mediated changes in SLM also have been related to UV-B protection (Balakumar *et al.*, 1993; Murali and Teramura, 1986a).

A number of drought-tolerant plant species are adapted to other forms of stress such as elevated temperatures and high photosynthetically active radiation. This has led to suggestions that drought (or general stress-) tolerance might also confer resistance to UV-B (Gwynn-Jones *et al.*, 1999b). To date this hypothesis has not been tested comprehensively. A study of two distant plant species (broad bean and wheat) suggested that growth advantages for wheat under UV-B were linked to higher drought tolerance in the latter species (Al-Oudat *et al.*, 1998).

Functional types are sets of plants displaying similar responses to environmental conditions (Diaz and Cabido, 1997). However, constraints due to species specificity do not allow ready generalisation of plant functional types based on UV-B responsiveness (Björn *et al.*, 1997) and it appears that more conclusive inferences can only be drawn from large-scale comparisons between species. Alternatively, it is possible to use comparisons among several populations of the same species, which are not compounded by between-species differences.

There are indications for UV-B tolerance in populations from habitats with higher ambient UV-B levels (Pukacki and Modrzynski, 1998). However, at present there is little information available on other relationships between UV-B responsiveness and features inherent to plants and their habitat, both on the species and on the population level. This precludes attempts to classify UV-B responsiveness according to functional types and is further complicated by the fact that most studies do not provide information about the environmental and physiological background of the investigated plant material (e.g. tolerance/sensitivity to drought, low temperature, shade) (Gwynn-Jones *et*

al., 1999b). To predict effects of UV-B on vegetation it would be valuable to have a framework that recognised underlying plant specialisations determining UV-B responsiveness. This work is a step towards such a framework by examining between-population differences in plant attributes and habitat adaptation for white clover and relating these to the sensitivity of those populations to UV-B.

The study examined genotypic differences among nine white clover populations to test several morphological and growth responses to UV-B. Detailed background information of plant and habitat features for these populations have been described in Chapter 3 (Table 3-1). Plants were exposed to a combination of 12 weeks UV-B and four weeks drought. In addition to aboveground productivity, particular emphasis was placed on leaf morphology and leaf initiation in the apical bud, complemented by measurements of stolon elongation rates. Two different water regimes were included to test the hypothesis that UV-B responses would be less pronounced under drought. It was further hypothesised that morphological differences among the white clover populations would be linked to their UV-B sensitivity.

Materials and Methods

Experimental design, plant material and cultivation, UV-B radiation treatment and application of drought have been described in Chapter 3. All plant measurements reported here were conducted at the conclusion of the 12 week UV-B exposure period (final harvest). Droughted plants were harvested the day after rewatering in order to prevent collection of wilted material.

Biomass production and leaf attributes

Aerial biomass was dried for 48 h at 80°C and subsequently weighed to calculate the rate of dry matter production. Sensitivity to UV-B was expressed as the percentage change calculated from the comparison of dry matter production between UV-B-treated and control plants. Leaf size was measured at the end of the experiment with a LI-COR Model 3100 area meter on four fully open distal leaves per plant. Laminae were subsequently dried at 80°C for 48 h for dry mass determination. The ratio of dry mass

over leaf area was calculated to give specific leaf mass (SLM), while leaf dry mass percentage (PDM) was calculated from (dry mass/fresh mass)*100.

Stolon growth and apical bud dissection

Shortly before onset of the drought period, ten stolons per population and treatment condition were marked at the node of a fully open distal leaf (leaf developmental stage 1.0 (Carlson, 1966)). At final harvest, the marked stolons were collected and stolon length and number of nodes determined in order to calculate stolon elongation rate (SER) and leaf appearance rate (LAR) per day. A subset of white clover populations ('Kopu', 'Huia' and 'Tienshan') were investigated in detail during dissection studies, examining leaf primordia formation in the apical bud. Examinations were conducted using a Nikon SMZ-2T stereoscopic dissecting microscope with lighting supplied by a Schott KL 1500 electronic cold-light source through fibre optic cables. Leaf primordia and their stipules were sequentially removed with a scalpel until the apical meristem was exposed, surrounded by the youngest primordia. The number of leaf primordia in the apical bud was determined for five replicate samples per population under each treatment condition.

Statistical analysis

Statistical analysis was performed with the General Analysis of Variance procedure in GENSTAT (Genstat, 1993), using either raw values or values transformed to a square-root or logarithmic scale. The GENSTAT Regression Analysis and Correlation procedures were used to examine relationships of plant attributes and UV-B sensitivity at final harvest. In an earlier study significant differences were established among the nine white clover populations under well-watered conditions for constitutive productivity (i.e. biomass production without UV-B supplementation) as well as for UV-B sensitivity (Chapter 3, see Fig. 3-2a). However, these earlier examinations revealed no population differences for UV-B sensitivity in plants exposed to drought. Correlations to aspects of constitutive biomass production and UV-B sensitivity were thus conducted on well-watered plants.

Results

In well-watered conditions, UV-B significantly reduced leaf size and leaf mass by about 15% across white clover populations, while plant productivity was reduced approximately by 20% (Table 4-1). This was different ($P < 0.05$) from observations under drought where the productivity decrease was marginal at about 8% while no significant UV-B-generated changes could be found for leaf size and leaf mass (Table 4-1). Leaf appearance rate (LAR, -5%) and stolon elongation rate (SER, -17%) also revealed significant overall reductions in well-watered conditions, but no change under drought (Table 4-1). Across white clover populations, specific leaf mass (SLM) did not change significantly under UV-B in either moisture treatment, and UV-B-elicited percent leaf dry mass (PDM) reduction by about 2% was marginally significant ($P = 0.078$) under drought (Table 4-1).

Compared to the UV-B-induced changes, drought affected morphology to a stronger degree than UV-B in all attributes measured (Table 4-1). Across UV-B treatments and white clover populations, significant ($P < 0.001$) decreases were recorded for productivity (-70%), leaf size (-50%), leaf mass (-25%), LAR (-25%) and SER (-60%), while SLM and PDM similarly significantly ($P < 0.001$) increased by 50% and 40%, respectively, in response to moisture deficit.

Dissection of the stolon tip in a subset of white clover populations revealed decreases for leaf initiation in the bud by an average 4% under UV-B and by 10% in response to drought ($P < 0.05$, Fig. 4-1).

Table 4-1. Attributes of growth and morphology (mean ± 1 SE) in white clover grown with (UV+) and without (UV-) supplementation of 13.3 kJ m^{-2} d^{-1} UV-B, under well-watered (W+) or drought conditions (W-). Probabilities reflect significance of the UV-B effect under the two water regimes. $P < 0.001$ = ***; $P < 0.01$ = **; $P < 0.05$ = *; $P < 0.10$ = $^{+}$, $P \geq 0.10$ = n/s. LAR = leaf appearance rate; PDM = percent leaf dry mass; SER = stolon elongation rate; SLM = specific leaf mass.

Attribute	Water	UV-	UV+	*P*
Productivity (mg day^{-1})	W+	353.1 ± 22.04	269.0 ± 13.37	***
	W -	103.6 ± 4.14	94.3 ± 2.87	$^{+}$
Leaf size (cm^2)	W+	8.12 ± 0.798	6.90 ± 0.677	***
	W -	3.68 ± 0.267	3.51 ± 0.292	n/s
Leaf mass (mg)	W+	17.6 ± 1.93	15.4 ± 1.69	**
	W -	11.8 ± 0.95	11.2 ± 1.01	n/s
SLM (mg cm^{-2})	W+	2.12 ± 0.034	2.18 ± 0.027	n/s
	W -	3.17 ± 0.041	3.15 ± 0.036	n/s
PDM (%)	W+	12.8 ± 0.17	12.6 ± 0.18	n/s
	W -	18.1 ± 0.20	17.7 ± 0.19	$^{+}$
LAR (day^{-1})	W+	0.256 ± 0.0043	0.242 ± 0.0050	*
	W -	0.183 ± 0.0044	0.183 ± 0.0045	n/s
SER (mm day^{-1})	W+	3.32 ± 0.165	2.75 ± 0.154	*
	W -	1.22 ± 0.073	1.15 ± 0.067	n/s

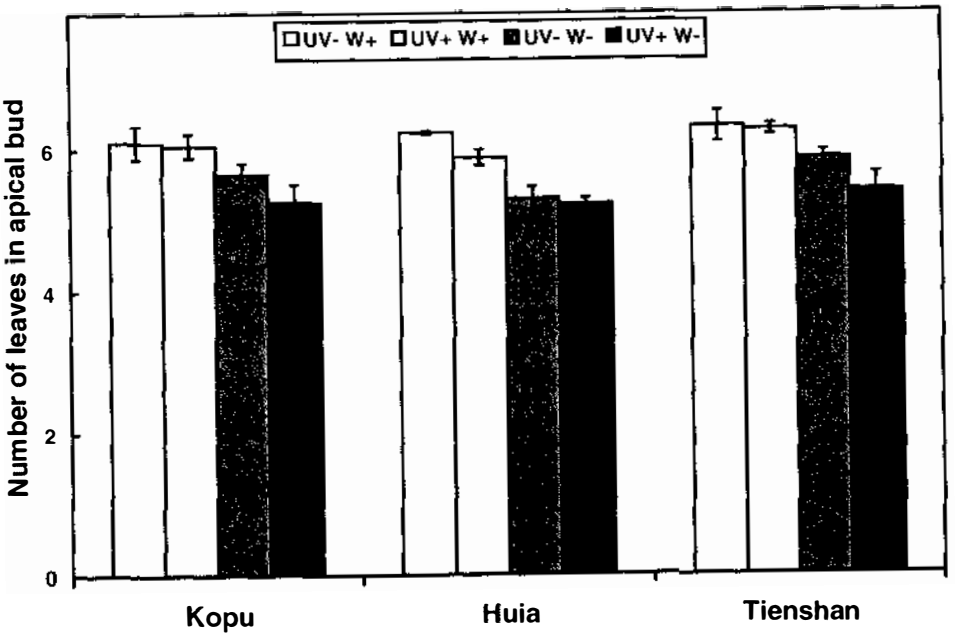


Fig. 4-1. Leaf initiation in apical buds of three white clover populations after 12 weeks supplementation with (UV+) or without (UV-) 13.3 kJ m^{-2} d^{-1} UV-B, under well-watered (W+) or drought conditions (W-). Error bars are ± SE.

Between-population comparisons

Previously, significant differences were reported in the productivity and UV-B sensitivity of the nine white clover populations under investigation, with UV-B tolerance for slower-growing, stress-tolerant ecotypes such as 'Tienshan', 'Sarikamis' and 'Häggås' (Chapter 3, Fig. 3-2a). In the present study there were significant intrinsic differences among the white clover populations for all leaf attributes ($P < 0.01$). For example, 'Octoploid', 'Haifa' and 'Kopu' were characterised by large leaf sizes (Fig. 4-2) and low PDM (Fig. 4-3), when compared to less productive white clover populations. However, response functions among white clover populations were more similar than within populations for the leaf measurements, thus preventing detection of significant within-species differences in foliar UV-B responses. 'Tienshan', 'Sarikamis' and 'Häggås' were more tolerant to UV-B in their stolon elongation rate under well-watered conditions, and together with 'Kopu' and 'Octoploid' this was significantly ($P < 0.05$) different from the SER reductions in the remaining populations (Fig. 4-4).

At the conclusion of the experiment, UV-B sensitivity in the nine white clover populations was related to higher constitutive productivity (Fig. 4-5a). Morphological attributes that were associated with both aspects of white clover growth in this study included leaf size and PDM. Large leaf size was associated with higher constitutive productivity (Fig. 4-5b) and also tended to relate to UV-B sensitivity ($r = 0.588$, $P = 0.096$). Significance for the latter correlations was also maintained under UV-B. PDM showed inverse relationships under UV-B to plant productivity (Fig. 4-5c) and to UV-B sensitivity ($r = -0.636$, $P = 0.066$). Productivity under drought was directly related to subtle increases in SLM ($r = 0.690$, $P < 0.05$).

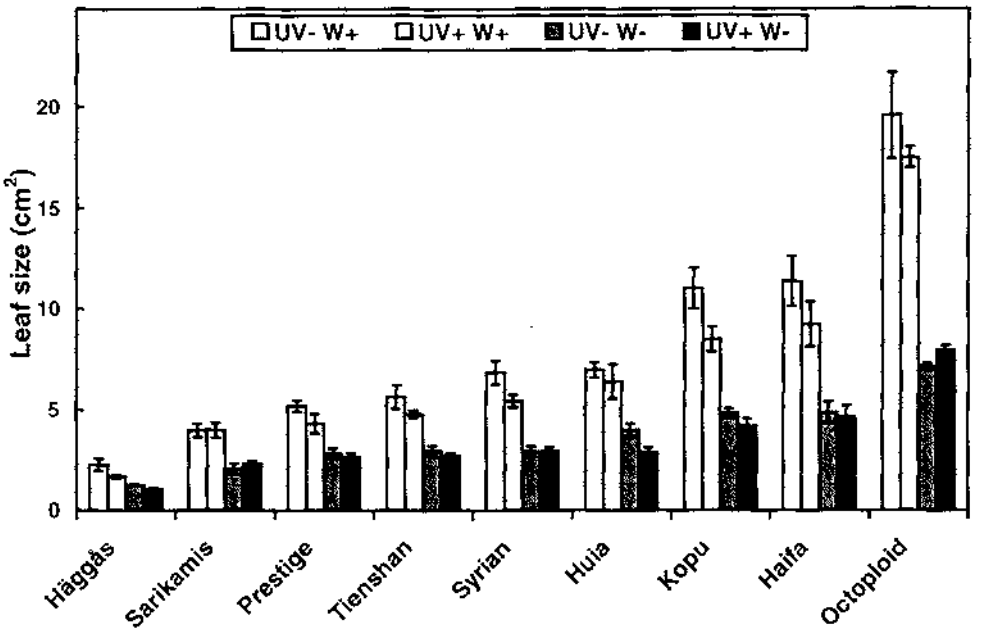


Fig. 4-2. Leaf size in nine white clover populations grown with (UV+) or without (UV-) supplementation of 13.3 kJ m^{-2} d^{-1} UV-B, under well-watered (W+) or drought conditions (W-). The populations are ranked by the leaf size of unstressed plants (UV- W+). Error bars are ± SE.

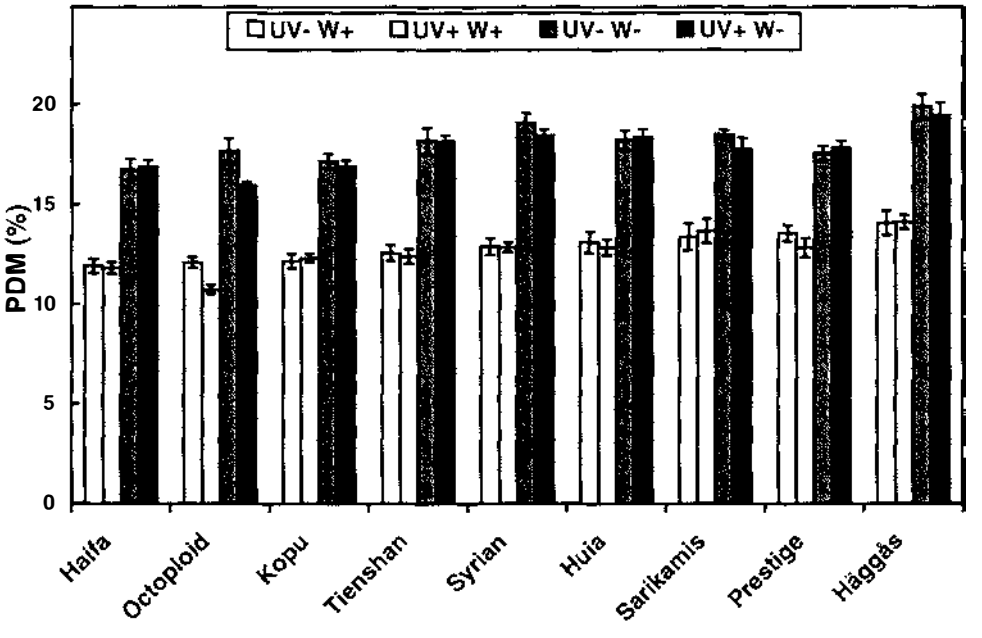


Fig. 4-3. Percentage leaf dry mass (PDM) in nine white clover populations grown with (UV+) or without (UV-) supplementation of 13.3 kJ m^{-2} d^{-1} UV-B, under well-watered (W+) or drought conditions (W-). The populations are ranked by the PDM of unstressed plants (UV- W+). Error bars are ± SE.

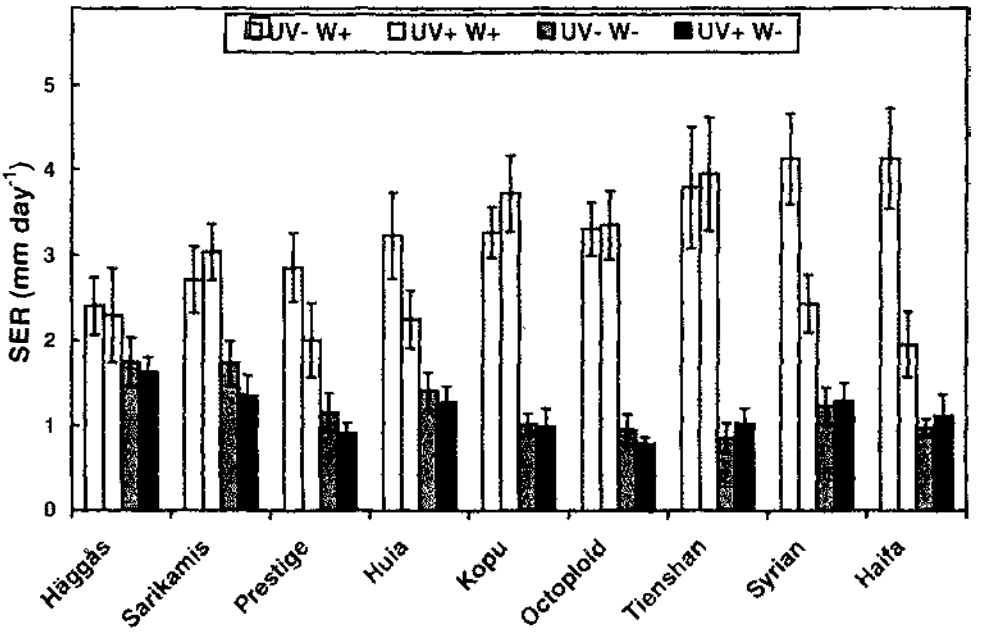


Fig. 4-4. Stolon elongation rate (SER) in nine white clover populations grown with (UV+) or without (UV-) supplementation of 13.3 kJ m^{-2} d^{-1} UV-B, under well-watered (W+) or drought conditions (W-). The populations are ranked by the SER of unstressed plants (UV- W+). Error bars are ± SE.

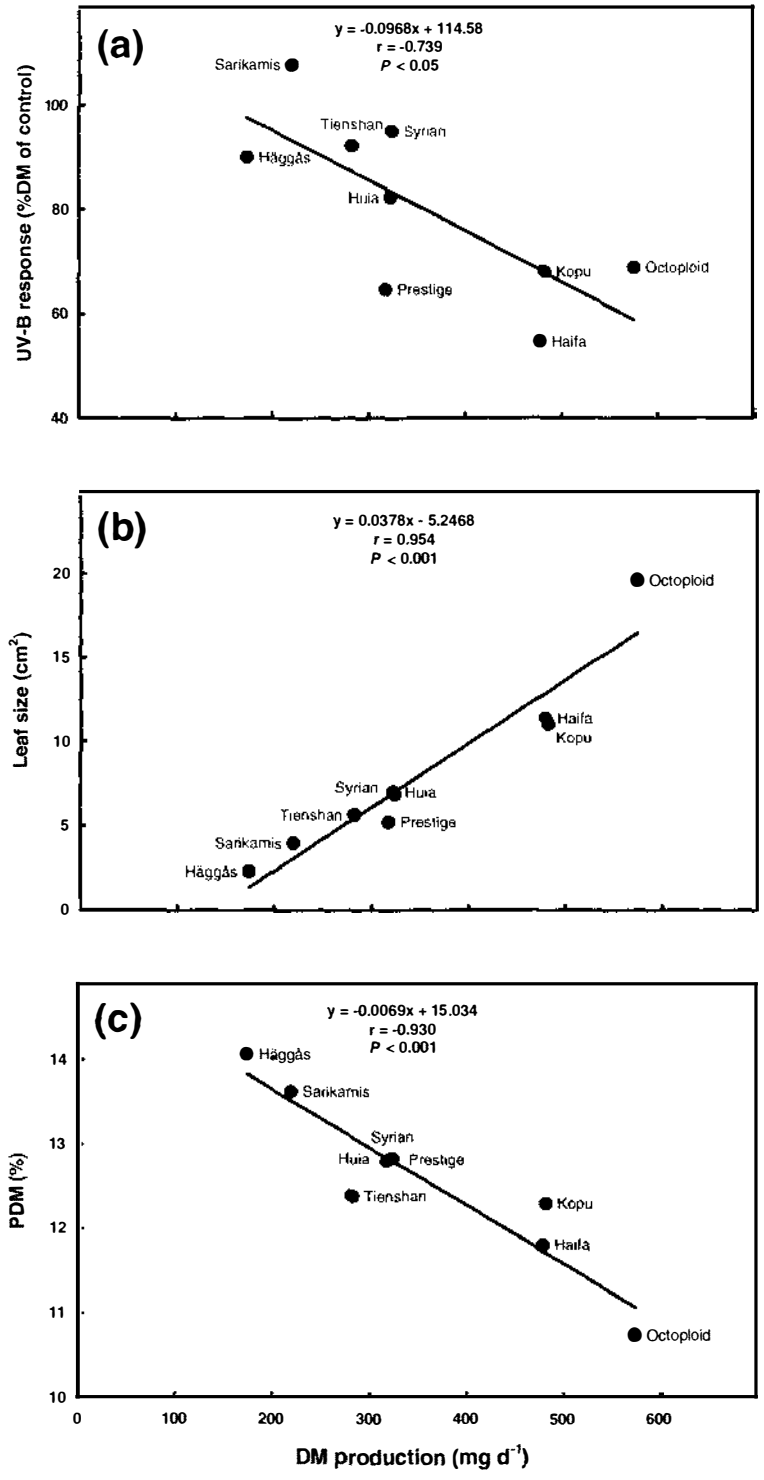


Fig. 4-5. Relationships of the constitutive productivity of nine white clover populations, measured as biomass production after 12 weeks growth under well-watered conditions without UV-B supplementation, to UV-B sensitivity and leaf attributes. (a) Relative growth, i.e. dry matter production after supplementation for 12 weeks with 13.3 kJ m^{-2} d^{-1} UV-B, expressed as a percentage of that of the control; (b) constitutive leaf size; (c) percentage leaf dry mass (PDM) under UV-B. DM = dry matter.

Discussion

Leaf and stolon morphology

This study revealed morphological changes that could affect plant and ecosystem processes (Rozema *et al.*, 1997c). Significant UV-B-induced reductions in overall aboveground productivity under well-watered conditions were accompanied by decreases in all measured morphological features contributing to biomass production (leaf size, leaf mass, leaf number and stolon elongation) (Table 4-1). Simultaneous decreases in total biomass accumulation and leaf area have been reported in a number of other studies (Correia *et al.*, 1999b; Li *et al.*, 1998; Nogues *et al.*, 1998). A field study of white clover also revealed similar UV-B-induced decreases in leaf size (Matthew *et al.*, 1996). In addition, UV-B-generated reductions in leaf appearance (LAR) were observed under well-watered conditions (Table 4-1). In a study on pea, leaf size was decreased by UV-B, while leaf number only was significantly reduced by drought (Nogues *et al.*, 1998). Leaf number reductions due to UV-B have been observed in *Phlomis fruticosa* (Nikolopoulos *et al.*, 1995). The observed reductions in both leaf size and leaf number suggest that reduced biomass accumulation results from reduced net carbon gain in both aspects contributing to total plant leaf area.

Apical bud dissections in three white clover populations show for the first time decreases in leaf primordia formation under UV-B (Fig. 4-1). This represents a developmental stage largely characterised by cell division (Thomas, 1987b). Previously it was demonstrated that UV-B-induced growth reductions were accompanied by decreased initiation in the number of apical buds (Chapter 2, Fig. 2-1) and the present findings suggest that detrimental UV-B effects on growth processes also occur inside the remaining buds. UV-B-induced changes in regions concealed from direct exposure have also been reported in pea, with reductions of leaf area and cell division in young leaves shielded against UV-B by folded bracts (Nogues *et al.*, 1998). Rather than direct effects, these findings suggest indirect modes of UV-B action resulting in reduced cell division in well-shielded plant parts, for instance decreased photosynthate supply or systemic responses through signal transduction and plant growth substances (Meijkamp *et al.*, 1999; Perennes *et al.*, 1999).

The present study also revealed detrimental UV-B effects for the elongation of white clover stolons under well-watered conditions (SER, Table 4-1 and Fig. 4-4). Reduced internode elongation in response to UV-B has also been observed in other studies (Ballare *et al.*, 1995; Gonzalez *et al.*, 1998; Hoffmann, 1999). Plant height was reduced due to UV-B in *Brassica* under well-watered, but not droughted conditions (Conner and Zangori, 1998). White clover stolon growth predominantly occurs horizontally and partially shaded by the leaf canopy and the observed decreases in elongation could further suggest systemic UV-B effects for plant growth. Similar to the findings from apical bud dissection, the observed reduction in stolon elongation could be related to reduced cell division. Reduced internode elongation in pea has been related to a decrease in cell numbers (Gonzalez *et al.*, 1998). Other studies also point at UV-B-induced destruction of IAA, again resulting in reduced elongation growth (Huang *et al.*, 1997).

Drought appeared to ameliorate or remove UV-B sensitivity in attributes of plant growth and morphology (Table 4-1). It has been argued that UV-B-irradiated plants might be advantaged under drought due to reduced water uptake as a consequence of UV-B-induced area reductions (Nogues *et al.*, 1998). However, in drought conditions, leaf size and leaf number did not differ significantly between UV-B treatments (Table 4-1), suggesting that such a mechanism was not operative here. Other studies also found UV-B-generated decreases in total plant biomass accumulation and leaf growth under well-watered conditions but a masking effect for such responses under drought (Murali and Teramura, 1986a; Sullivan and Teramura, 1990; Teramura *et al.*, 1990). It is possible, that mechanisms linked to reduced biomass accumulation under moisture limitation already provide grounds for UV-B protection. Such protective function could be mediated by drought via delays in cell division and expansion, thus reducing the chance of damage by UV-B on these processes. Growth delay has been described as an important UV-B-protective mechanism, particularly when growth of the particular organ is to a significant extent due to cell division (Beggs *et al.*, 1986). This would be supported by observations of significant drought effects on processes linked to cell division in the apical bud (Fig. 4-1).

Relative leaf dry mass allocation

A different response pattern was observed in attributes of relative leaf dry mass allocation (specific leaf mass, SLM and percent leaf dry mass, PDM) (Table 4-1). UV-B had a similar effect on leaf size and leaf mass and thus no significant UV-B-induced change could be found for SLM under either water regime. Other studies found increases for SLM in response to UV-B (Li *et al.*, 1998; Tosserams and Rozema, 1995), while - similar to the present findings - SLM was unaffected by UV-B irrespective of water supply in studies on soybeans (Murali and Teramura, 1986b; Sullivan and Teramura, 1990). Long-term studies in *Phlomis fruticosa* (Nikolopoulos *et al.*, 1995) and investigations of 38 plant species in Mediterranean-type ecosystems (Wand, 1995) also detected no change in SLM under elevated UV-B, while PDM was slightly decreased in the latter study (Wand, 1995).

Nevertheless, strong increases for SLM and PDM were observed under drought (Table 4-1 and Fig. 4-3), concomitant with a decrease in overall UV-B sensitivity when moisture supply was limited (Table 4-1). This has also been reported in other studies, suggesting involvement of drought-generated SLM increases for UV-B tolerance (Balakumar *et al.*, 1993; Murali and Teramura, 1986a). Thus, in addition to a passive response to drought via reduced cell division, active drought-induced morphogenetic functions could contribute to UV-B protection, e.g. via increases in relative leaf dry mass allocation. Increased SLM can represent increased thickness or higher density of the leaf and protective functions for higher SLM have been related to improved leaf UV-B absorption properties (Britz and Adamse, 1994). While small changes in SLM under UV-B did not appear to relate to UV-B tolerance, it is possible that only a marked increase in SLM is of relevance for UV-B protection.

Between-population comparisons

Differential UV-B sensitivity of the white clover populations was related to their higher constitutive productivity (Fig. 4-5a). Populations bred for agricultural yield were more affected by UV-B than unselected, wild ecotypes of lower productivity and from stress-exposed habitats (Chapter 3, Fig. 3-2a). It was therefore of interest whether such constitutive and UV-B-induced differences in growth would be related to morphological

features. It was found that more productive white clover populations had larger leaves with higher relative moisture content (lower PDM, especially under UV-B) (Fig. 4-5b,c) and these attributes in turn tended to correlate with UV-B sensitivity. Thus plant features contributing towards resource acquisition also showed some direct relationships to UV-B sensitivity. Previously, a relationship has been reported between differential leaf size and sensitivity to drought among white clover populations (Barbour *et al.*, 1996). Smaller constitutive leaf size and higher PDM in the UV-B-tolerant white clover ecotypes can also be seen as an adaptive characteristic to multiple stress conditions in their habitat of origin. These ecotypes derive from habitats with low annual precipitation of 700mm or below, which would be considered marginal levels for white clover growth (Hoglund and White, 1985; Jones and Lowe, 1993). In other studies, the Asian and Scandinavian ecotypes were distinct from white clover cultivars originating in warmer, moister climates such as 'Huia' on the basis of lower yield, smaller leaves and low cyanogenesis (Caradus and Forde, 1996; Caradus *et al.*, 1989b).

Lower PDM is reflective of higher relative leaf moisture content which in turn is related to leaf protein content and ultimately higher growth rates (Wilson *et al.*, 1999). This is also confirmed by the relationship between PDM and productivity observed in the present study (Fig. 4-5c). No such relationship could be found for SLM, and other studies have also pointed at higher relative importance for leaf dry matter content in the prediction of stress tolerance, rather than SLM which can be influenced by a number of leaf-internal variables (Wilson *et al.*, 1999). Nevertheless it was of interest to note that productive populations showed small UV-B-induced increases in SLM under drought. While not related to UV-B tolerance, this could still reflect some morphological adaptation in higher yielding white clover populations. UV-B sensitivity in the higher-yielding cultivars could be also related to increased genome size or activity (Campbell and Grime, 1993). This seems supported by UV-B sensitivity observed in 'Octoploid', containing double the usual tetraploid white clover chromosome set.

Conclusions

From these results taken overall, the observed responses can be divided into two distinct categories. The first type of response is represented by decreased morphological development under UV-B in well-watered conditions, accompanied by further drought-mediated reductions without an additional UV-B effect (Fig. 4-2 and Fig. 4-4). The second response pattern is that of a pronounced drought-induced increase in morphogenetic aspects of relative leaf dry matter allocation, largely independent of UV-B (Fig. 4-3). These two response classes are similar to main means of UV-B protection described for plants (Beggs *et al.*, 1986), namely minimising damage by growth delay and by structural attenuation.

The present work addresses a recently expressed need to classify UV-B responsiveness according to functional types (Gwynn-Jones *et al.*, 1999b). It has been stated that UV-B sensitivity in crop plants is related to the plant breeding process, selecting (often in glasshouses eliminating UV-B radiation) for attributes that potentially could confer UV-B sensitivity such as high growth rates, thin leaves, reduced secondary compounds and elimination of structural features affecting palatability (Rozema *et al.*, 1997c). Elite germplasm producing high yields is usually adapted to a narrow set of environmental conditions and does not perform well under more marginal environmental conditions (Groth, 1998). The studies in white clover go beyond the species level, showing for the first time that within-species differences in UV-B responsiveness can be related to different functional population types, separated by attributes linked to plant and habitat productivity. A trade-off between investment in competitive strategy and UV-B susceptibility proposes ecological relationships under UV-B that have previously been established for other forms of stress (Grime and Campbell, 1991; Poorter and Garnier, 1999). Comprehensive testing will be required to examine such relationships on a between-species level.

Chapter 5. Effects of ultraviolet-B radiation on *Trifolium repens* physiology: mediation by drought and relationships to UV-B sensitivity

Water potential measurements

An abbreviated version of this Chapter has been submitted to *Plant, Cell and Environment.*

Abstract

This study investigated functional responses of white clover to UV-B in interaction with drought. A particular aim was to relate these responses to previously established differences in white clover sensitivity to UV-B. Nine white clover populations were used, ranging from highly productive cultivars to slow-growing ecotypes from stress-exposed habitats. Using controlled environmental conditions, plants were exposed in a factorial design to supplementation with or without ultraviolet-B radiation (UV-B) of 13.3 kJ m^{-2} d^{-1} for 12 weeks, accompanied by four weeks of drought. Examinations included accumulation of UV-absorbing compounds, flavonoids and chlorophylls as well as several measures of photosystem II function. Measurements linked to plant water status included leaf water potential and free proline accumulation. Flavonoids were identified as glycosides of the flavonols quercetin and kaempferol. UV-B increased the levels of UV-absorbing compounds and flavonol glycosides and this was synergistically enhanced in combination with drought. These responses were more pronounced for quercetin than for kaempferol glycosides. Drought alone did not increase flavonol glycoside accumulation. Chlorophyll levels and photochemical parameters were not negatively affected by UV-B. UV-B increased leaf water potential by 16% under drought, and proline levels by 23% in well-watered conditions. Differences in these responses due to water treatment and white clover population revealed that increases in UV-absorbing compounds, higher quercetin glycoside accumulation and increased water potential under UV-B can confer UV-B tolerance in drought and are characteristic of slow-growing, UV-B-tolerant populations adapted to multiple stress.

Introduction

New Zealand pasture ecosystems are experiencing elevated ultraviolet-B radiation (UV-B, 290-315 nm) as a result of stratospheric ozone loss (McKenzie *et al.*, 1999). White clover serves as the principal nitrogen-donating component in these ecosystems and has shown susceptibility both to UV-B and drought (Barbour *et al.*, 1996; Matthew *et al.*, 1996), stress forms that frequently coincide in the natural environment. Drought-induced water stress influences most physiological processes via turgor reductions and decreases in cell division as well as cell expansion (Hsaio, 1973). UV-B effects on

various aspects of plant function have also been demonstrated (Rozema, 1999; Tevini, 2000). Furthermore, drought modifies physiological responses to UV-B (Nikolopoulos *et al.*, 1995; Teramura *et al.*, 1983; Tevini *et al.*, 1983a).

While UV-B can act as a stress affecting primary plant metabolism, it is also seen as a regulatory factor affecting secondary plant processes, for example via effects on phenolic compounds (Rozema *et al.*, 1999). Several studies suggest UV-B-protective functions for phenylpropanoid derivatives such as hydroxycinnamic acids and flavonoids (Markham *et al.*, 1998b; Meijkamp *et al.*, 1999; Olsson *et al.*, 1998). Some studies attribute the primary role for flavonoid induction to UV-B (Allen *et al.*, 1999; Balakumar *et al.*, 1993), while other reports suggest an interaction with drought (Nogues *et al.*, 1998). Comparisons between and within species show that higher UV-absorbing compound accumulation can be associated with plants from habitats with elevated natural UV-B background levels (Rozema *et al.*, 1997a; Wand, 1995).

A number of studies have also investigated UV-B effects on photosynthesis and photochemistry in conjunction with drought. UV-B-elicited changes on chlorophyll levels and on the chlorophyll a:b ratio can be independent of water supply (Eswaran *et al.*, 1993; Teramura *et al.*, 1984b), but synergistic interactions have also been demonstrated (Nikolopoulos *et al.*, 1995). Other reports show population-specific differences in chlorophyll content under UV-B (Murali and Teramura, 1986b). Chlorophyll fluorescence studies have frequently been used to study UV-B effects on photosystem II (PSII) function. Intrinsic efficiency of PS II (F_v/F_m) in dark-adapted leaves is proportional to apparent photochemical quantum yield and often decreases when plants are stressed. Investigations of UV-B effects on the photochemical efficiency of PSII in combination with drought showed UV-B-induced decreases (Nikolopoulos *et al.*, 1995), no change (Allen *et al.*, 1999), or even increases of F_v/F_m (Manetas *et al.*, 1997; Petropoulou *et al.*, 1995). UV-B stress in PSII after light adaptation has been observed in the form of reduced photochemical yield ($\Delta F/F'_m$) (Reuber *et al.*, 1996a). Reduced photochemical efficiency under UV-B has also been measured as decreased photochemical quenching (q_P) (Reuber *et al.*, 1996b). Photochemical UV-B stress can also be reflected by changes in non-photochemical quenching (NPQ), giving an indication of energy dissipation as heat (Reuber *et al.*,

1996a). Studies in rye showed UV-B-generated decreases in both photochemical and non-photochemical quenching (Reuber *et al.*, 1996b). There are also reports of population-specific effects of UV-B on PS II activity (Reuber *et al.*, 1996a). Compared to the tolerant mother variety, a UV-B sensitive barley mutant displayed reduced F_v/F_m and $\Delta F/F'_m$ as well as increases in q_P under UV-B (Reuber *et al.*, 1996a), while NPQ showed UV-B-induced decreases in both populations (Reuber *et al.*, 1996a).

Several studies have investigated UV-B effects on plant water relations. UV-B-generated improvements have been observed for relative water content and leaf water potential (ψ_L) under drought in pea (Nogues *et al.*, 1998). Studies in soybean also showed that UV-B effects on leaf water relations can depend on moisture availability (Teramura *et al.*, 1984a). Accumulation of free proline has been related to protection against a number of environmental factors, including drought, salt, temperature and heavy metal stress (Naidu, 1998; Saradhi *et al.*, 1995). However, few studies have investigated proline responses to UV-B. A study in *Phaseolus mungo* reported proline increases under UV-B (De Britto, 1995). Proline levels have shown insensitivity to UV-B in well-watered cowpea plants but were decreased by UV-B in drought conditions (Balakumar *et al.*, 1993).

More than 20 studies have investigated UV-B effects in combination with drought, but information about physiological mechanisms underlying the resulting growth responses is less than comprehensive (Nogues *et al.*, 1998). There is a need for studies examining constitutive and UV-B-induced functional attributes that can potentially interact with both forms of stress. A beneficial effect of the UV-B × drought combination for growth responses has been related to drought-induced stimulation of UV-absorbing compound accumulation (Murali and Teramura, 1986a) and to increased leaf water content via increases in leaf cuticle thickness under UV-B (Manetas *et al.*, 1997). Very little functional information is available about stress interactions in conjunction with populations of the same species (Krupa *et al.*, 1998a).

In particular it has yet to be tested comprehensively whether adaptation to other forms of stress can be linked to UV-B tolerance (Gwynn-Jones *et al.*, 1999b). This could be expected from other studies on stress interactions using between-species comparisons

(Grime *et al.*, 1997). In Chapter 3 and Chapter 4 it was demonstrated that detrimental UV-B effects on white clover growth occurred in well-watered conditions, and that these were mitigated under concurrent drought. These studies also showed that UV-B sensitivity under well-watered conditions was related to the productivity of the white clover populations and of their habitat of origin. The present study aims to investigate whether these differences in productivity and UV-B sensitivity can be related to physiological differences among the white clover populations. This would contribute to the establishment of a predictive framework that relates plant specialisations to UV-B responsiveness. To this effect these studies utilised the significant potential for population differences within white clover.

Nine white clover populations from different genetic and geographical backgrounds were exposed to 12 weeks of UV-B and four weeks of drought. This was done in growth rooms to exclude variability in environmental conditions that would normally interfere in the investigation of processes underlying plant responses to UV-B (Corlett *et al.*, 1997). Characteristics of the white clover populations have been outlined in more detail in Chapter 3 (Table 3-1). The study examined accumulation of UV-absorbing compounds, flavonoids and chlorophylls as well as several chlorophyll fluorescence parameters. The application of moisture deficit also warranted examination of aspects related to plant water status, including measurements of ψ_L and of free proline levels. The drought treatment further allowed testing the hypothesis that functional UV-B responses in white clover would be altered by moisture deficit. This was done to discern possible mechanisms underlying amelioration of the UV-B growth response established previously. A further aim was to compare functional aspects in the white clover populations to the previously observed within-species differences in plant productivity and UV-B-induced growth responses. It was hypothesised that white clover populations tolerant to conditions of stress in their habitats of origin would display a higher degree of biochemical or physiological adaptation to UV-B.

Materials and Methods

Experimental design, plant material and cultivation, UV-B radiation treatment and application of drought have been described in Chapter 3. All plant attributes reported

here were measured at the conclusion of the 12 week UV-B exposure period (final harvest). Droughted plants were harvested the day after rewatering in order to prevent collection of wilted material.

UV-absorbing compounds

UV-absorbing compound levels were estimated by established procedures (Mirecki and Teramura, 1984). After oven-drying, samples of four fully open distal trifoliate laminae were ground and weighed (15-20 mg) into 1.5 ml centrifuge tubes. Extraction in 1.2 ml MeOH:H_2O:HCl (79:20:1) was performed in darkness for 24 h under occasional shaking. This was followed by centrifugation and spectrophotometric analysis of the supernatant in 3 ml quartz cuvettes. Absorbance readings at 300 nm were calculated on the basis of leaf dry weight. This procedure was repeated on five replicates for each white clover population under each treatment condition.

Flavonoid analysis

For flavonoid analysis, 1.5-2 g of fully open trifoliate laminae were collected from each pot. This yielded five replicate samples for each of the nine populations at the two treatment levels. Immediately after collection, samples were frozen in liquid nitrogen and stored at -80°C. Each sample was subsequently ground in liquid nitrogen using a mortar and pestle. The resulting fine leaf powder was freeze-dried and stored at -80°C until analysed. The dried, ground plant material (50-60 mg) was extracted with occasional vortex shaking for 24 h in 3 ml MeOH:H_2O:HOAc (89:10:1), followed by centrifugation and de-fatting of the supernatant with 1 ml petroleum ether. After solvent evaporation under nitrogen followed by vacuum drying, the residue was taken up into 700 µl CH_3CN:HOAc:H_2O (32:3:65). The solution was centrifuged and a 25µl sample was used for HPLC analysis.

Analytical HPLC was conducted using a Jasco PU-980 Intelligent HPLC solvent delivery system, Waters 994 programmable photodiode array detector and a Gilson 234 autosampler. Chromatography was carried out on a Merck Supersphere Lichrocart 125-4 RP-18 endcapped column (4 µm, 4 mm x 119 mm) with a gradient solvent system

comprising solvent A [1.5% H_3PO_4] and solvent B [HOAc:CH_3CN:H_3PO_4:H_2O (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% at 39.3 min. Peaks of quercetin and kaempferol derivatives were identified on the basis of the on-line spectra. The integrated areas of all flavonol peaks (measured at 352 nm) were added to calculate flavonol glycoside levels from a standard curve prepared using rutin (quercetin 3-rutinoside).

Chlorophyll concentration

Extraction of chlorophyll from fully open white clover laminae was performed in N, N-dimethylformamide (DMF) (Moran and Porath, 1980). Three samples for each population under each treatment condition were ground, freeze-dried and weighed (10 mg) into centrifuge tubes. Samples were extracted with 1.2 ml DMF in darkness at 4°C for 24 h under occasional vortex shaking. This was followed by centrifugation at 20 000 *g* and 4°C for 3 min. Absorbance of the supernatant was read at 664.5 nm and 647 nm and chlorophyll a and b content was calculated using the following equations (Inskeep and Bloom, 1985):

Chlorophyll a = 12.7 $A_{664.5}$ - 2.79 A_{647}

Chlorophyll b = 20.7 A_{647} - 4.62 $A_{664.5}$

Total Chlorophyll = 17.9 A_{647} + 8.08 $A_{664.5}$

Chlorophyll fluorescence

Chlorophyll fluorescence measurements were performed on fully open distal leaflets of 4-5 replicate samples for each population under each treatment condition (Greer, 1995a; Greer, 1995b). Samples were stored on moist filter paper immediately after collection in cuvettes sealed with a rubber stopper. Dark-adapted samples were collected before onset of the light period and had been in darkness for at least 10 h. Using a PAM fluorometer at room temperature (Pam 101; Walz, Effeltrich, Germany), initial fluorescence in darkness F_o was measured using a measuring light. Following this, maximum chlorophyll fluorescence in darkness F_m and the fluorescence ratio F_v/F_m ($F_v = F_m - F_o$), indicating intrinsic efficiency of photosystem II (PS II) 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 before measurements were conducted for light-adapted initial (F_t) and maximum (F_m') fluorescence and actual efficiency of PS II during illumination (photochemical yield = $\Delta F/F'_m$). Photochemical (q_P) and non-photochemical (NPQ) quenching were calculated, where $q_P = (F_m' - F_t)/(F_m' - F_o)$ and NPQ = $(F_m - F_m')/F_m'$.

Leaf water potential and proline

Leaf water potential (ψ_L) was determined using the pressure chamber technique (Scholander *et al.*, 1965; Turner, 1981). Fully open distal leaves were enclosed in a pressure chamber and the cut surface of the protruding petiole was monitored while pressure in the chamber was gradually increased. The pressure required to bring xylem water to the surface (equivalent to the water tension inside the xylem) was recorded. Measurements were conducted on five replicate samples per population and treatment condition.

Levels of free proline in fully open white clover laminae were determined using established methods (Magne and Larher, 1992). For each population under each treatment condition, three samples were ground in liquid nitrogen, freeze-dried and 10 mg weighed into centrifuge tubes. Precipitation of protein was done in 1.2 ml of 3% (w/v) sulphosalicylic acid under vortex shaking, followed by centrifugation at 12 000 *g* for 7 min. The supernatant was removed and after renewed centrifugation 500 µl were collected and made up to 1 ml with water. This was followed by addition of 2.0 ml ninhydrin reagent (1% [w/v] ninhydrin in 60% [v/v] glacial acetic acid), vortex shaking and reaction of the solution for 1 h at 98°C. The reaction was stopped in an ice-water bath, followed by addition of 2 ml toluene and vortex shaking for 20 sec. Phases were allowed to separate for at least 5 min and the products extracted in toluene were examined spectrophotometrically in a 1 ml glass cuvette at 520 nm. Free proline content in the white clover samples was calculated from a standard curve of known proline concentrations (0 to 25 µg/ml) prepared in an identical manner alongside each batch of samples.

Statistical analysis

The same statistical approaches were used as described in Chapter 4.

Results

UV-absorbing compounds and flavonoids

UV-absorbing compound levels displayed an overall increase in response to UV-B ($P < 0.001$). This effect was most pronounced in drought-stressed plants, with a significantly ($P < 0.05$ for the UV-B × drought interaction) greater average change (+12%) than well-watered plants (+3%) (Fig. 5-1). Changes in UV-absorbing compound levels under UV-B showed differences among the white clover populations ($P < 0.05$), with particular increases for the ecotypes 'Tienshan' and 'Sarikamis' across water treatments (Fig. 5-1).

Flavonoids in the white clover leaf samples were identified by HPLC analysis as glycosides of the flavonols quercetin and kaempferol. Significant UV-B-induced increases in flavonol glycosides ($P < 0.001$) differed between water treatments ($P < 0.001$ for the UV-B × drought interaction). Drought-induced changes alone showed no consistent pattern and flavonol glycoside levels on average were similar to unstressed conditions. Flavonol glycoside accumulation in the white clover populations in response to UV-B was consistently higher than under unstressed conditions or under drought alone (Fig. 5-2). Compared to unstressed conditions, a more than double increase of flavonol glycoside levels was observed under UV-B, and the increase was more than triple in the UV-B × drought combination across populations (Fig. 5-2).

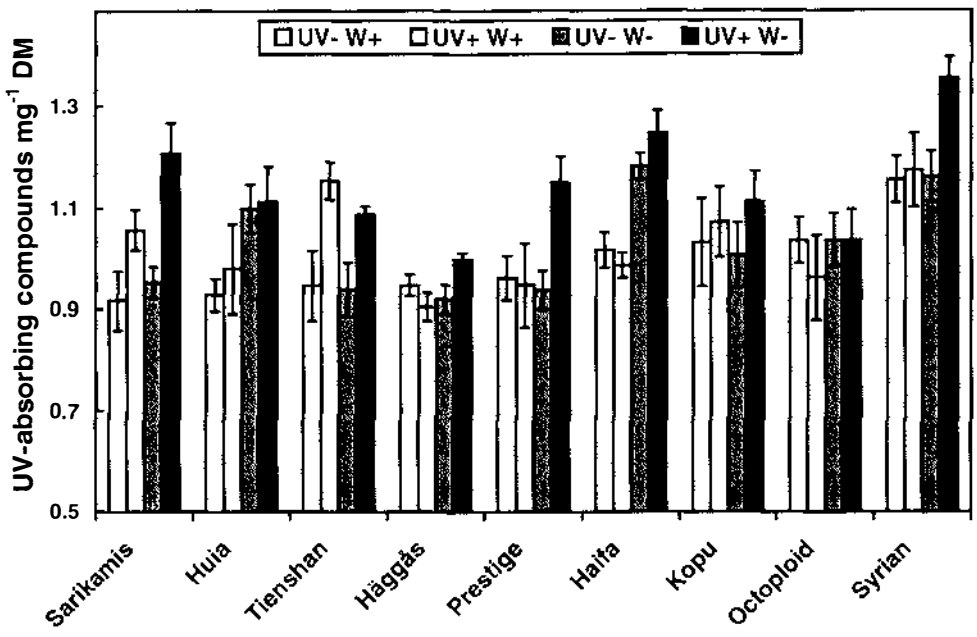


Fig. 5-1. UV-absorbing compounds (at 300 nm) per mg leaf dry mass (DM) in nine white clover populations grown with (UV+) and without (UV-) supplementation of 13.3 kJ m^{-2} d^{-1} UV-B, under well-watered (W+) or drought (W-) conditions. The populations are ranked by the levels of unstressed plants (UV- W+). Error bars are ± SE

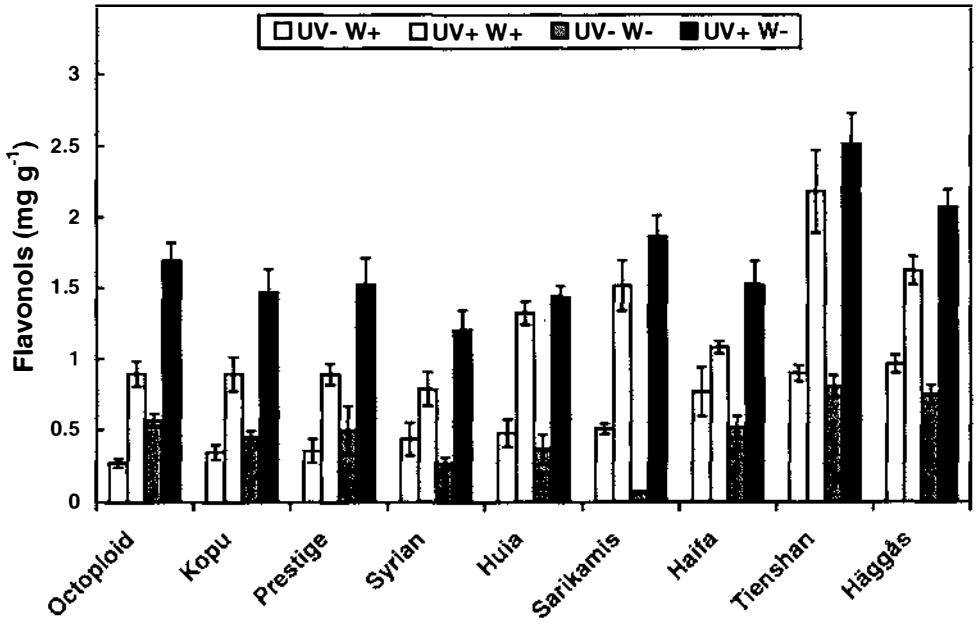


Fig. 5-2. Flavonol glycoside levels per g leaf dry mass in nine white clover populations grown with (UV+) and without (UV-) supplementation of 13.3 kJ m^{-2} d^{-1} UV-B, under well-watered (W+) or drought conditions (W-). The populations are ranked by the flavonol glycoside accumulation of unstressed plants (UV- W+). Error bars are ± SE.

Furthermore, there were significant differences in the UV-B-induced flavonol glycoside responses among white clover populations ($P < 0.001$). Particularly high flavonol glycoside accumulation was observed for the white clover ecotypes 'Tienshan', 'Sarikamis' and 'Häggås' (Fig. 5-2). These responses were also observed for individual flavonols, especially quercetin glycosides, which under UV-B showed higher absolute levels and larger increases than kaempferol compounds (Table 5-1). Accumulation of individual flavonol glycosides in the nine white clover populations has been further detailed in Chapter 6, but see also Fig. 5-5a here. The UV-B-generated changes in UV-absorbing compound levels were related to the UV-B responses in flavonol glycoside accumulation ($r = 0.698$, $P < 0.05$).

Photosynthetic pigmentation and photochemistry

Photosynthetic pigmentation experienced little change in response to UV-B (Table 5-1). UV-B led to a marginal increase in chlorophyll levels by 4% under well-watered conditions and to no change in the chlorophyll a:b ratio. Similarly, no marked UV-B effects could be measured for chlorophyll fluorescence and related quenching factors, with a small 3% UV-B-generated increase in photochemical yield ($\Delta F/F'_m$) under both watering regimes the only significant change (Table 5-1). Drought had a stronger effect with an average 17% decrease in chlorophyll levels ($P < 0.01$) and a 35% increase for non-photochemical quenching (NPQ, $P < 0.001$). No significant interactions between UV-B and drought or white clover populations could be found for chlorophyll levels or any of the photochemical measurements.

Table 5-1. Biochemical and photochemical attributes (mean ± 1 SE) in white clover grown with (UV+) and without (UV-) supplementation of 13.3 kJ m^{-2} d^{-1} UV-B, under well-watered (W+) or drought conditions (W-). Probabilities reflect significance of the UV-B effect under the two water regimes. $P < 0.001$ = ***; $P < 0.05$ = *; $P < 0.10$ = $^{+}$, $P \geq 0.10$ = n/s. $\Delta F/F_m$ = photochemical yield; F_v/F_m = intrinsic efficiency of photosystem II; NPQ = non-photochemical quenching; q_P = photochemical quenching.

Attribute	Water	UV-	UV+	*P*
Quercetin (mg g^{-1})	W+	0.26 ± 0.031	0.77 ± 0.049	***
	W -	0.13 ± 0.019	1.05 ± 0.048	***
Kaempferol (mg g^{-1})	W+	0.30 ± 0.028	0.47 ± 0.035	***
	W -	0.35 ± 0.034	0.65 ± 0.040	***
Chlorophyll (mg g^{-1})	W+	15.36 ± 0.225	15.98 ± 0.306	$^{+}$
	W -	13.04 ± 0.242	13.32 ± 0.191	n/s
Chlorophyll a:b ratio	W+	3.13 ± 0.014	3.12 ± 0.014	n/s
	W -	3.17 ± 0.016	3.14 ± 0.021	n/s
F_v/F_m	W+	0.842 ± 0.0017	0.843 ± 0.0014	n/s
	W -	0.847 ± 0.0016	0.848 ± 0.0014	n/s
$\Delta F/F_m$	W+	0.626 ± 0.0042	0.639 ± 0.0027	*
	W -	0.620 ± 0.0035	0.639 ± 0.0025	***
q_P	W+	0.905 ± 0.0083	0.922 ± 0.0084	n/s
	W -	0.966 ± 0.0094	0.978 ± 0.0088	n/s
NPQ	W+	0.938 ± 0.0307	0.970 ± 0.0313	n/s
	W -	1.332 ± 0.0387	1.274 ± 0.0387	n/s

Leaf water potential and proline

There was a significant ($P < 0.001$) UV-B effect on leaf water potential (ψ_L). The greater overall increase occurred under drought (16%), which was significantly ($P < 0.001$) different from the overall 3% increase for ψ_L under well-watered conditions (Fig. 5-3). This compared to a pronounced average decrease of about 50% for ψ_L under drought ($P < 0.001$) (Fig. 5-3). There were significant differences among well-watered white clover populations in the constitutive (i.e. UV-B-independent) ψ_L ($P < 0.01$) and in the degree of the UV-B-induced changes for ψ_L ($P < 0.05$). In particular, increases in ψ_L could be observed for 'Tienshan' and 'Sarikamis' and decreases for 'Haifa' and 'Octoploid' (Fig. 5-3). A direct relationship between constitutive ψ_L and UV-B-generated ψ_L changes ($r = 0.729$, $P < 0.05$) showed that populations with low intrinsic ψ_L were able to increase their ψ_L under UV-B, while less negative constitutive values for ψ_L were linked to UV-B-elicited decreases in ψ_L (Fig. 5-3).

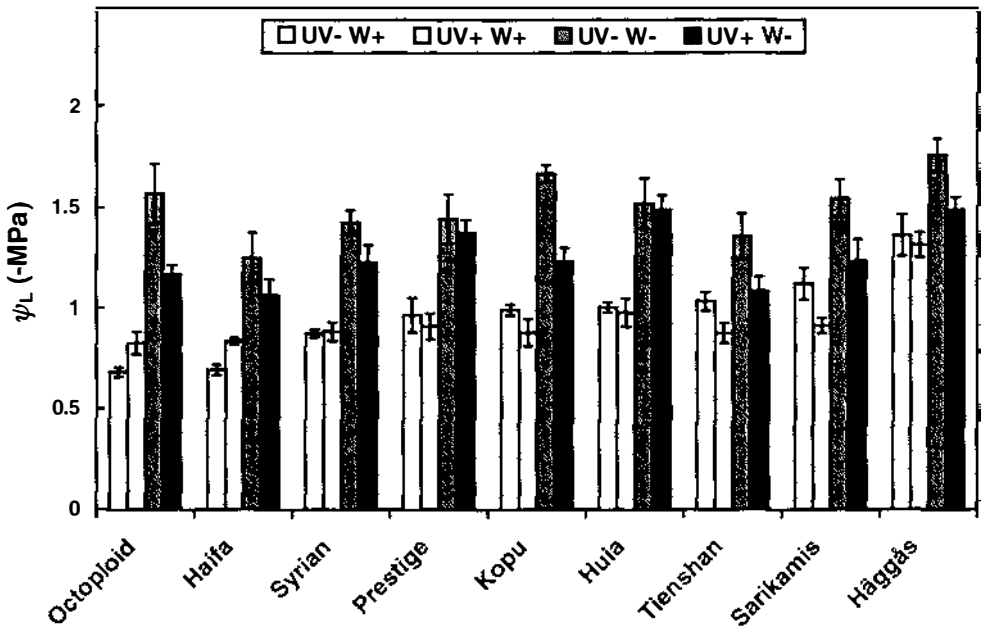


Fig. 5-3. Leaf water potential (ψ_L) in nine white clover populations grown with (UV+) and without (UV-) supplementation of 13.3 kJ m^{-2} d^{-1} UV-B, under well-watered (W+) or drought conditions (W-). The populations are ranked by the ψ_L of unstressed plants (UV- W+). Error bars are ± SE.

Free proline levels were consistently enhanced by UV-B under well-watered conditions, leading to an overall increase by 23% ($P < 0.01$) (Fig. 5-4a). Compared to the well-watered plants, drought led to a 30-fold increase in proline accumulation independent of UV-B treatment. Averaged across white clover populations, there was no significant overall difference for free proline accumulation in response to UV-B under drought. Nevertheless there were differences in this response among the white clover populations ($P < 0.05$), in particular with decreases in proline accumulation for 'Syrian' and 'Sarikamis' (Fig. 5-4b). Significant inverse relationships were found between intrinsic proline concentration (i.e. without UV-B supplementation) and increases in these levels under UV-B ($r = -0.867$, $P < 0.01$ for well-watered and $r = -0.708$, $P < 0.05$ for droughted plants). This showed that in both water regimes free proline concentrations were more strongly increased by UV-B in white clover populations that had low intrinsic proline levels (Fig. 5-4a,b). Populations with low ψ_L contained high proline levels under UV-B ($r = -0.694$, $P < 0.05$) in drought conditions and a similar relationship could also be found for well-watered plants ($r = -0.610$, $P = 0.081$). Under

well-watered conditions, there was also an inverse constitutive relationship between ψ_L and quercetin glycoside levels (r = -0.730, $P < 0.05$).

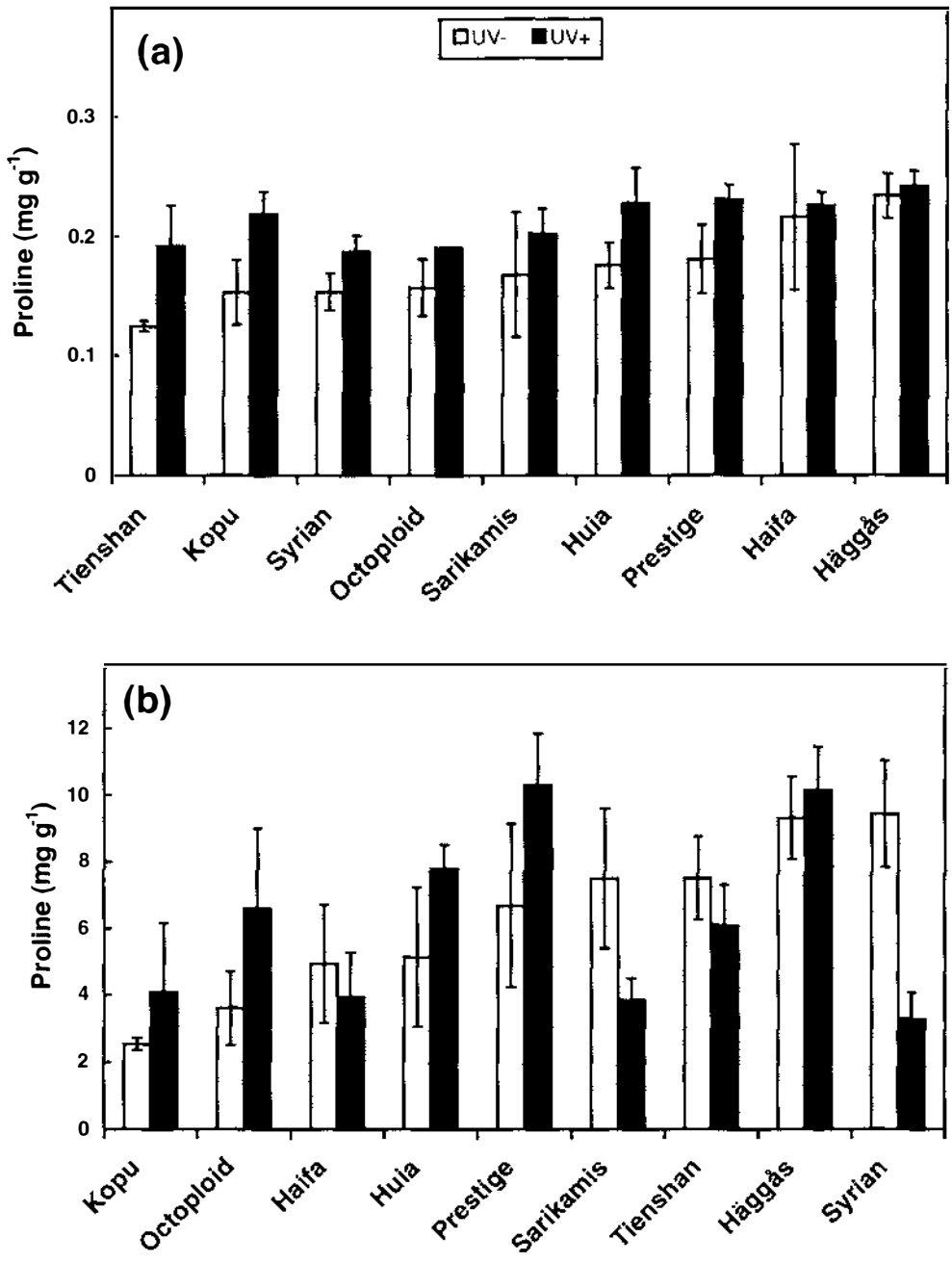


Fig. 5-4. Proline accumulation per g leaf dry mass in nine white clover populations grown with (UV+, closed columns) and without (UV-, open columns) supplementation of 13.3 kJ m^{-2} d^{-1} UV-B in (a) well-watered conditions, and (b) under drought. Under both watering regimes the populations are ranked by the proline concentration of plants not exposed to UV-B supplementation (UV-). Error bars are ± SE.

Intraspecific relationships to biomass production and UV-B sensitivity

Earlier studies showed that UV-B sensitivity in the nine white clover populations was related to higher constitutive productivity (Chapter 3, Fig. 3-3 and Chapter 4, Fig. 4-5a). Physiological factors that were associated with both aspects of white clover growth in this study included quercetin glycoside accumulation and ψ_L. Productivity of the white clover populations under well-watered conditions showed an inverse relationship to constitutive (r = -0.826, $P < 0.01$) and UV-B-induced accumulation of quercetin glycosides (Fig. 5-5a) and this in turn was linked to UV-B tolerance (r = 0.629, P = 0.069). No relationships to productivity or UV-B tolerance could be found for kaempferol glycosides.

Similarly under well-watered conditions, productive populations had higher (less negative) constitutive ψ_L (Fig. 5-5b), and this in turn tended to be inversely related to UV-B sensitivity (r = -0.605, P = 0.085). Productive populations decreased their ψ_L most under UV-B (Fig. 5-5c) and this decrease again showed an inverse trend towards UV-B sensitivity (r = -0.637, P = 0.065). Furthermore, UV-B tolerance in the white clover populations correlated with the capacity to increase accumulation of UV-absorbing compounds under UV-B (Fig. 5-6), and a similar relationship was found for increases in flavonol glycoside levels (r = 0.588, P = 0.096). In drought conditions there was a significant inverse correlation of proline concentration with plant productivity (r = -0.728, $P < 0.05$), showing that the less productive ecotypes contained the highest proline levels (Fig. 5-4b).

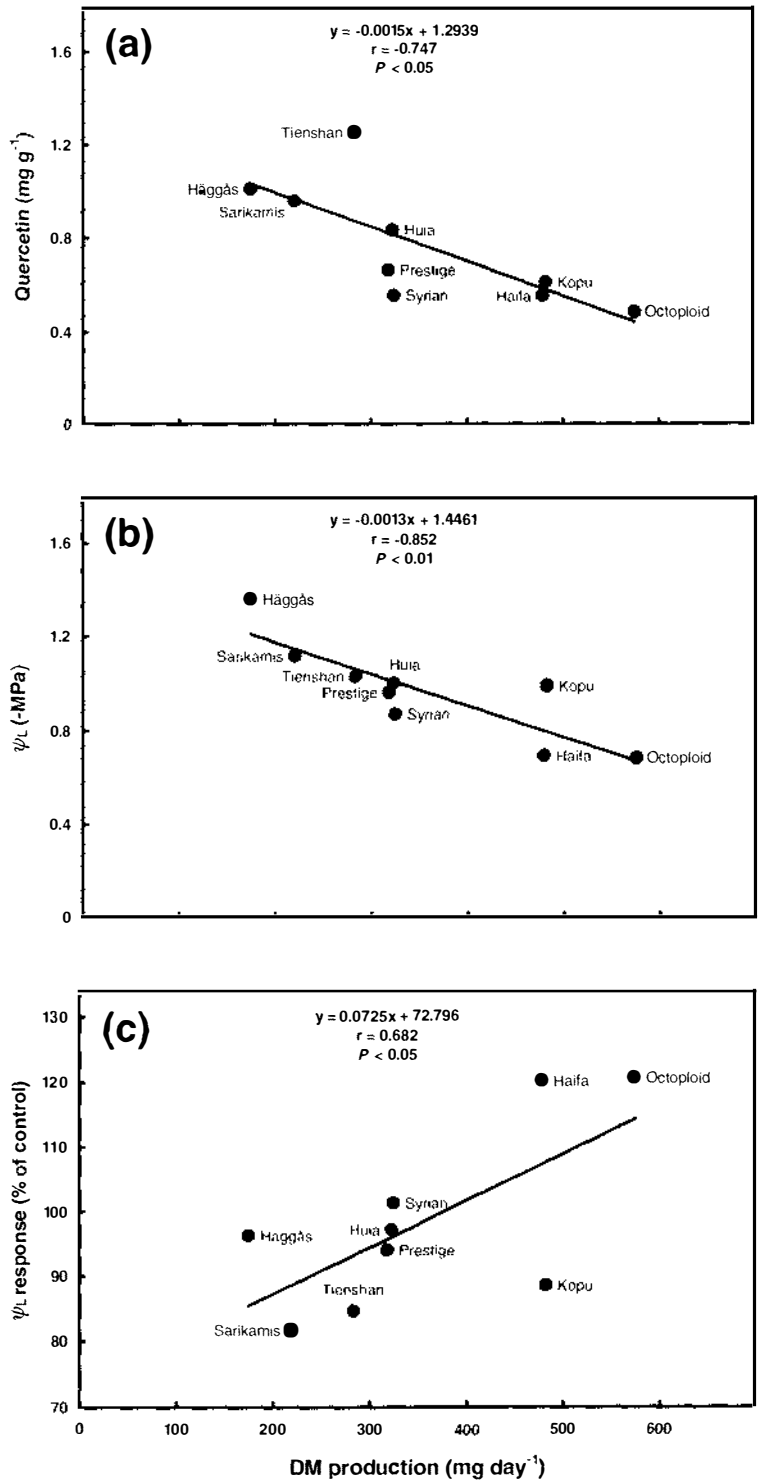


Fig. 5-5. Relationships of the constitutive productivity of nine white clover populations, measured as biomass production after 12 weeks growth under well-watered conditions without UV-B supplementation, to (a) quercetin glycoside accumulation under UV-B (b) constitutive leaf water potential (ψ_L) and (c) relative change in ψ_L (value under UV-B expressed as a percentage of that of the control) in response to elevated UV-B of 13.3 kJ m^{-2} d^{-1} for nine white clover populations. DM = dry matter.

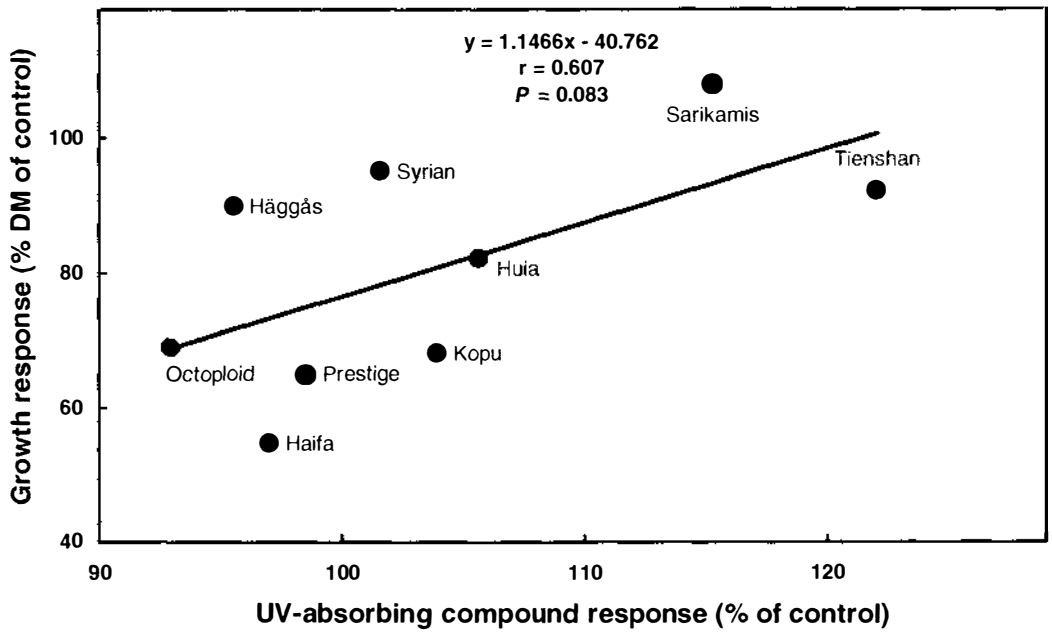


Fig. 5-6. Relationship between UV-B-induced relative changes (values under UV-B expressed as a percentage of the control) in aboveground dry matter production and in UV-absorbing compounds (measured at 300 nm) for nine white clover populations grown under well-watered conditions with or without UV-B supplementation of 13.3 kJ m^{-2} d^{-1}. DM = dry matter.

Discussion

UV-absorbing compounds and flavonoids

A number of significant physiological changes were observed in response to UV-B which were dependent on water availability and differed among white clover populations. This included UV-B-induced increases in UV-absorbing compound and flavonoid levels, and a synergistic response in the combination of UV-B and drought (Fig. 5-1 and Fig. 5-2).

UV-B-induced increases in UV-absorbing compound levels have been noted frequently, reflecting the general regulatory role of UV-B (Rozema *et al.*, 1999). Increases for UV-absorbing compounds particularly in the combination of UV-B and drought have also been observed in pea (Nogues *et al.*, 1998) and in the Mediterranean plant *Cistus creticus* (Stephanou and Manetas, 1997). The findings clearly indicate that white clover could require combination with drought for pronounced UV-absorbing compound accumulation under UV-B. There were significant population differences in UV-B responsiveness among the white clover populations, with UV-absorbing compound increases in the ecotypes 'Tienshan' and 'Sarikamis' in both water treatments (Fig. 5-1). These populations originate in habitats exposed to multiple stresses and it is possible that adaptation to these also facilitated acclimation to UV-B.

Similarly, there were significant differences in constitutive and UV-B-induced flavonol glycoside levels among the white clover populations with highest accumulation under UV-B in the stress-tolerant ecotypes 'Sarikamis', 'Tienshan' and 'Häggås' (Fig. 5-2). Comparison with the results for UV-absorbing compound accumulation shows similar overall direction, but much more pronounced quantitative changes for flavonol glycoside levels in response to UV-B. This is in accord with other studies, reporting underestimation of the flavonoid response in the crude UV-absorbing compound extraction which contains flavonoids and other phenolics as well as compounds not involved in the UV-B response (Meijkamp *et al.*, 1999). There are suggestions that other UV-B-protective phenolics such as hydroxycinnamic acids frequently contribute towards a constitutive shield against UV-B, while flavonoid accumulation often undergoes strong induction by UV-B (Bornman *et al.*, 1997; Meijkamp *et al.*, 1999).

A primary role of UV-B for the enhancement of flavonol glycoside in white clover is indicative of the specific roles these compounds fulfil in UV-B protection, including UV-B absorption (Markham, 1982), radical scavenging (Yamasaki *et al.*, 1997), energy dissipation (Smith and Markham, 1998) and growth control (Meijkamp *et al.*, 1999). Similar UV-B-induced enhancement of flavonoid levels and synergistic interaction with drought have been reported in radish (Tevini *et al.*, 1983a) and in *Cistus ladanifer* (Chaves *et al.*, 1997). The interactive role of moisture deficit with UV-B for flavonol glycoside accumulation observed here could be due to activation of plant regulatory factors common to both forms of stress, resulting in the combined up-regulation of phenylpropanoid pathway genes (Tevini, 1994).

In unstressed plants, equal amounts of quercetin and kaempferol glycosides were found (Table 5-1). However, quercetin clearly increased more than kaempferol glycosides under UV-B (Table 5-1). Such preferential UV-B-elicited accumulation of B-ring *ortho*-dihydroxylated flavonoids over their closely related monohydroxylated counterparts has been reported in a number of recent studies, (e.g. Markham *et al.*, 1998a; Markham *et al.*, 1998b; Olsson *et al.*, 1998). Higher quercetin glycoside accumulation in stress-adapted white clover ecotypes (Fig. 5-5a) could be reflective of a general role for B-ring *ortho*-dihydroxylated flavonoids in plant stress responses. Dihydroxylated flavonoids can be more efficient for UV-B protection in comparison to their monohydroxylated counterparts due to higher antioxidant activity (Montesinos *et al.*, 1995) and energy dissipation (Smith and Markham, 1998).

Earlier results showed significant reduction in white clover productivity by UV-B in well-watered conditions, and masking of this effect by drought (Chapter 3 and Chapter 4). The observed synergistic accumulation of flavonol glycosides and UV-absorbing compounds under UV-B and drought could counter UV-B-induced growth reduction that would normally occur under well-watered conditions. A study in soybean suggested possible interaction between UV-B and drought due to drought-generated increases in flavonoid levels which in turn were linked to protection against UV-B (Murali and Teramura, 1986a). A number of stress factors (e.g. temperature, drought, light, UV-B) can cause similar oxidative processes in plant tissues, suggesting that adaptation or

exposure to one stress can lead to tolerance against another (Balakumar *et al.*, 1993; Manetas *et al.*, 1997; Petropoulou *et al.*, 1995).

Photosynthetic pigmentation and photochemistry

Photosynthetic parameters in white clover were not negatively affected by UV-B, both on the basis of pigmentation and from a photochemical perspective. Other studies found increases (Visser *et al.*, 1997a), no effect (Tosserams and Rozema, 1995) and decreases (Correia *et al.*, 1999a) for chlorophyll levels in response to UV-B. While chlorophyll levels were not significantly affected by UV-B in field-grown soybeans, a synergistic increase in the chlorophyll a:b ratio could be detected (Murali and Teramura, 1986a). When measured across seasons, however, the chlorophyll a:b ratio remained unaffected by UV-B irrespective of water supply (Murali and Teramura, 1986b). Absence of UV-B or drought effects on chlorophyll fluorescence and other photosynthetic parameters in the presence of growth reductions have also been observed in other studies (Gonzalez *et al.*, 1996; Nogues *et al.*, 1998). Small increases in chlorophyll levels and photochemical yield ($\Delta F/F'_m$) (Table 5-1) could nevertheless indicate a subtle regulatory function of UV-B, although this remains uncertain considering the minor degree of the observed changes. The observation that chlorophyll content decreased under drought but not in response to UV-B further suggests a negligible role of the latter stress for photosynthetic pigmentation in white clover.

Maintenance of photosynthetic capacity has also been observed in pea plants developing from seed under UV-B (Nogues *et al.*, 1998). This may reflect adequate photoprotective mechanisms including the presence of UV-B-screening pigmentation outlined above. These observations suggest major physiological UV-B action occurs via indirect effects on processes linked to secondary metabolism, rather than processes related to primary photosynthetic efficiency. However, photosynthesis could still be inhibited without UV-B effects on PSII photochemistry or pigmentation, for example due to leaf area reductions or by effects on stomata, leading to altered plant-water relations and growth (Nogues *et al.*, 1998). Stomatal closure in response to UV-B has been observed and resulting stomatal limitations have been related to decreased photosynthetic productivity (Nogues *et al.*, 1999).

Leaf water potential and proline

Possible UV-B-induced stomatal limitations could become apparent in improved plant water relations, for instance higher (less negative) leaf water potential (ψ_L). This study revealed increases in ψ_L across white clover populations in the drought treatment and for some populations also under well-watered conditions, particularly for the stress-tolerant populations 'Tienshan' and 'Sarikamis' (Fig. 5-3). Another study demonstrated decreases in cell expansion under UV-B for white clover (Chapter 2, Table 2-2). The observed increases in ψ_L in the present study, however, suggest no negative UV-B effect on leaf cell turgor. This could point to other effects of UV-B on cell wall extensibility, e.g. via processes antagonistic to indole acetic acid function (Huang *et al.*, 1997).

UV-B-induced increases of the plant water status and of ψ_L particularly under drought have also been observed in pea (Nogues *et al.*, 1998), in *Pinus* (Manetas *et al.*, 1997) and in soybeans (Teramura *et al.*, 1984a). Such studies have related mutual beneficial effects of drought and UV-B for the plant water status to smaller leaf area, increased cuticle thickness and decreased stomatal conductance. Another study in white clover showed UV-B-induced increases in the root:shoot ratio (Chapter 2, Table 2-2), further providing possible morphogenetic means for the improvement in the physiological water status. Such improvements could be expected more important under limited moisture availability, and this may contribute to the observed increases for ψ_L in the combination of UV-B and drought.

Free proline levels increased markedly in response to water deficit, showing that plant metabolism had been changed by drought and demonstrating the established osmoprotective function for this amino acid (Naidu, 1998). However, there were also consistent UV-B-induced free proline increases under well-watered conditions (Fig. 5-4a). Studies in *Phaseolus mungo* demonstrated UV-B-induced changes in proline levels under well-watered conditions (De Britto, 1995). Using UV-C radiation, other studies also reported changes in proline levels, including decreases in maize (Brito *et al.*, 1997) and increases in rice, mustard and mung bean (Saradhi *et al.*, 1995).

However, changes under UV-C irradiation have to be regarded with caution, as this waveband can elicit markedly different responses to UV-B (Kozak *et al.*, 1999). Furthermore, the commonly used method for proline determination has been demonstrated open to carbohydrate interference (Magne and Larher, 1992). Using an approach addressing this, the present study demonstrates for the first time UV-B-induced enhancements in proline accumulation across a number of populations within a plant species.

Under drought there was no overall UV-B effect on free proline accumulation in the white clover plants. Nevertheless, significant population differences for proline levels could be observed, both with and without UV-B supplementation. High initial proline levels in the white clover ecotypes and in 'Syrian' under drought (Fig. 5-4b) could be genetic adaptations to the stress- and drought-exposed evolutionary background of these populations. Two of these populations, 'Syrian' and 'Sarikamis' displayed strong UV-B-induced decreases in proline concentration under UV-B (Fig. 5-4b), reflecting an inverse relationship between intrinsic proline levels and UV-B-induced responses. While strong variability in proline accumulation under dry conditions has been reported for white clover (Barker *et al.*, 1993), decreases in proline levels under the UV-B × drought combination have also been observed in cowpea (Balakumar *et al.*, 1993). This was related to a markedly improved water status under the combination of treatments (Balakumar *et al.*, 1993). In general it appears that white clover populations already high in intrinsic proline do not invest metabolic energy in further increases of free proline levels or even decrease them under UV-B.

A role for proline in the screening of UV-B is not likely, supported by examination of the proline absorption spectrum which is characterised by a lack of UV-absorbance (data not shown). Other studies have shown that proline can contribute towards protection from peroxidative processes resulting from enhanced UV-B (Saradhi *et al.*, 1995). While no direct relationship to UV-B protection could be observed for the subtle (compared to the drought effect) UV-B-generated enhancements in free proline levels, it can not be excluded that the strongly increased proline levels under drought contribute towards amelioration of UV-B sensitivity.

Drought-mediated physiological changes of potential beneficial value for UV-B protection in white clover can therefore be classified as follows. They can (1) either be largely mediated by drought alone (e.g. increases in proline accumulation) or (2) are dependent on the combination of drought with UV-B, including synergistic increases in UV-absorbing compounds, flavonol glycosides and ψ_L.

Intraspecific relationships to biomass production and UV-B sensitivity

Earlier studies revealed significant differences in UV-B sensitivity among the nine white clover populations and that this was related to their differential productivity (e.g. Chapter 3, Fig. 3-3). A further aim was thus to identify possible mechanisms underlying such differences among the populations. The correlation studies suggest inverse relationships of productivity and UV-B sensitivity in white clover populations with quercetin glycoside levels under UV-B (Fig. 5-5a). UV-B-generated increases in UV-absorbing compounds (Fig. 5-6) were also related to UV-B tolerance. A similar relationship of UV-absorbing compound accumulation with UV-B tolerance has been found in population comparisons in soybean (Murali and Teramura, 1986b) and in cucumber (Murali and Teramura, 1986c).

Several studies have described a relationship between origin from habitats high in natural UV-B (e.g. due to higher altitude or lower latitude) and higher epidermal UV-B attenuation or UV-absorbing compound accumulation (Larson *et al.*, 1990; Robberecht *et al.*, 1980; Rozema *et al.*, 1997a; Wand, 1995; Ziska *et al.*, 1993). Other studies found no relationships of UV-absorbing compound accumulation to altitudinal origin or growth rate in different *Poa* species (Pilon *et al.*, 1999). The population studies in white clover demonstrate for the first time a relationship between accumulation of specific flavonoid compounds and plant productivity (Fig. 5-5a). In particular, the less productive white clover ecotypes 'Tienshan', 'Sarikamis' and 'Häggås' accumulated higher amounts of quercetin glycosides under UV-B (Fig. 5-5a) and this in turn was linked to UV-B tolerance. No such relationships could be found for kaempferol glycosides. This specificity highlights that other processes apart from mere alteration of carbon budgets are involved in secondary metabolite accumulation of relevance for UV-B protection. The more productive populations are generally sensitive to a number of

stress forms, and also to UV-B (Chapter 3, Fig. 3-3). These data suggest that such general stress susceptibility is related to deficiencies in biochemical defense mechanisms, exemplified here by lower accumulation of flavonoid - and particularly quercetin - compounds.

The observed inverse constitutive relationship between accumulation of quercetin glycosides and plant productivity supports other views linking accumulation of such compounds to hormonal growth control via inhibition of auxin (Meijkamp *et al.*, 1999). While the slower growth of stress-tolerant ecotypes might be linked to higher intrinsic levels of specific flavonol compounds, it is also possible that these populations are better adapted to increased accumulation of such pigments under UV-B. This is further supported by ecological theory of stress tolerance, proposing higher biochemical adaptation for slower-growing plant species (Grime and Campbell, 1991). The present results confirm this on a population level, suggesting that strategies directing carbon flow preferentially towards biomass accumulation result in disadvantages for other carbon-requiring secondary metabolic functions of relevance for UV-B protection, including a general UV-absorbing compound response and the specific accumulation of flavonol glycosides. This also confirms suggestions of a particular role for the shikimate pathway in UV-B protection, linking sugar and flavonoid metabolism (Meijkamp *et al.*, 1999). It has frequently been stated that the metabolic costs of UV-B protection are unknown (Rozema *et al.*, 1999). The correlation studies could suggest that one metabolic cost of a plant strategy towards constitutive and UV-B-induced protection against UV-B may be lower investment of carbon allocation towards primary productivity.

Under well-watered conditions, less productive white clover populations had lower constitutive ψ_L (Fig. 5-5b) and this in turn tended to be linked to UV-B sensitivity. The higher ψ_L for productive populations in this study was reflected by higher morphological moisture status (lower PDM) in Chapter 4 (Fig. 4-3). Higher physiological water status thus reflects a strategy towards higher biomass accumulation, which in turn carries the morphological cost of higher UV-B sensitivity. Furthermore, an inverse relationship between quercetin glycoside accumulation and ψ_L under well-watered conditions could indicate that the lower ψ_L in less productive, UV-B-tolerant

white clover populations could at least partly be due to higher constitutive content of ψ_L-decreasing vacuolar solutes involved in UV-B protection.

This study further demonstrated that UV-B-generated changes in ψ_L can differ among plant populations. In particular, there were relationships of UV-B-generated changes in ψ_L with plant productivity (Fig. 5-5c) and UV-B responsiveness. Productive, more UV-B sensitive white clover populations (particularly 'Haifa' and 'Octoploid') experienced UV-B-generated decreases in ψ_L. Findings in cucumber relate UV-B sensitivity to UV-B-induced decreases in stomatal resistance, leading in turn to growth reductions via increased water stress (Teramura *et al.*, 1983). Increases in ψ_L for less productive, UV-B-tolerant white clover ecotypes ('Tienshan' and 'Sarikamis') could be related to the previously observed maintenance of root dry matter production and increased relative root mass (root:shoot ratio) under UV-B in these populations (Chapter 2, Fig. 2-1).

In drought conditions, free proline accumulation was inversely related to productivity, further suggesting osmoprotective adaptation under limited moisture supply in the lower-yielding ecotypes. The fact that increases in UV-absorbing compounds, but not proline levels show a positive relationship with UV-B tolerance points at the specificity of the biochemical UV-B response in these populations. Moreover, inspection of free proline accumulation under drought (Fig. 5-4b) shows that the slower-growing populations (already high in proline accumulation without UV-B) generally did not increase, or even decrease their proline levels under UV-B, while the opposite was found for accumulation of flavonol glycosides (Fig. 5-2). These findings could indicate a fundamental difference at the level of amino acid biosynthesis between production of the heterocyclic proline and that of the aromatic flavonoid precursors tyrosine and phenylalanine. The slower-growing populations appear to be more efficient in directing their aminocarbon flow towards the latter process under UV-B.

Conclusions

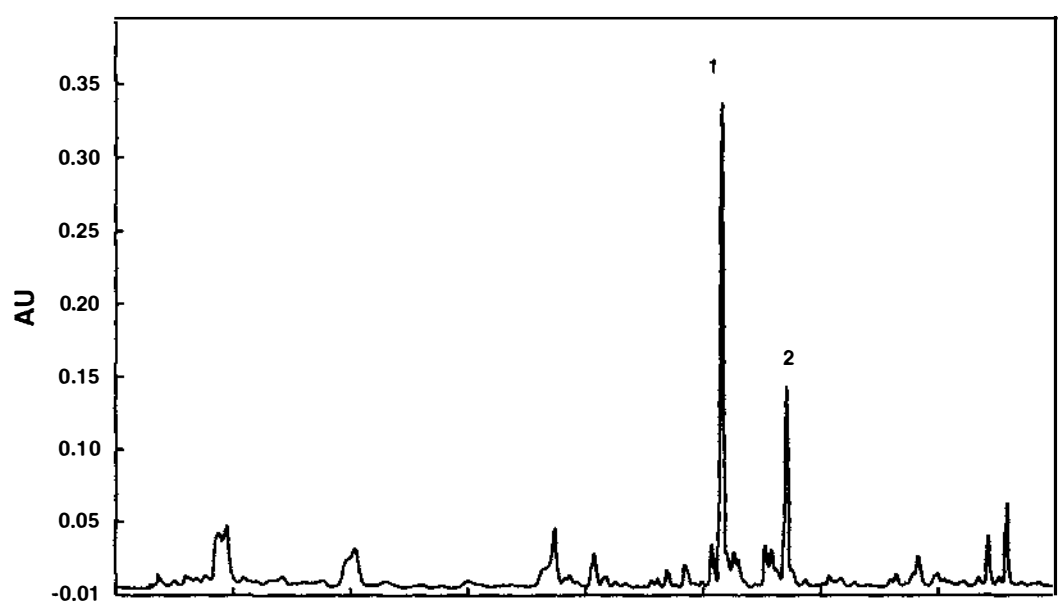
The results from population comparisons reinforce suggestions from drought-mediated physiological changes of potential beneficial value for UV-B protection. It appears that UV-B-induced increases in UV-absorbing compounds, higher quercetin glycoside

accumulation as well as increased ψ_L not only could contribute towards general amelioration of the UV-B growth response under drought, but even more importantly display on a population level and under well-watered conditions correlations with attributes of UV-B tolerance. This is of particular advantage for slow-growing, UV-B-tolerant populations adapted to multiple stress. Three classes of UV-B protection have been suggested, namely protection by growth delay, UV-B attenuation and repair mechanisms (Beggs *et al.*, 1986). The physiological changes observed here could fulfil roles in all three categories, particularly mediated by flavonol glycosides. These compounds could provide a 'priming effect' for tolerant populations via constitutive growth delay, while strongly increased flavonol glycoside levels, together with other UV-absorbing compounds, would reduce damage by UV-B attenuation and dissipation. Repair functions could be performed via possible antioxidant activities, particularly by quercetin glycosides.

UV-B tolerance in native, unbred plant species has been related to structural advantages (e.g. thick leaves) and higher levels of secondary compounds resulting from exposure to natural stress factors (Rozema *et al.*, 1997c). This study demonstrates on a population level that stress-adapted ecotypes of lower productivity have higher capacity for biochemical adaptation, which in turn confers protection against UV-B. The correlation studies (Fig. 5-5a,c and Fig. 5-6) and significant population differences in functional responses to UV-B suggest that UV-B tolerance is mainly related to UV-B-induced levels and changes in physiological characteristics. Examination of attributes of morphology and growth showed that it was generally constitutive, rather than UV-B-induced levels that were linked to UV-B responsiveness (Chapter 2, Fig. 2-2a,b and Chapter 4, Fig. 4-5a,b).

Taken together, this indicates the existence of functional population types in white clover. A conceptual framework is proposed, suggesting that (1) a general advantage of lower productivity is increased capacity for biochemical responses towards UV-B protection, while (2) plant types specialised towards biomass gain would display morphological responsiveness in the form of reduced productivity.

Chapter 6. Responses of nine *Trifolium repens* L. populations to ultraviolet-B radiation: differential flavonol glycoside accumulation and biomass production



HPLC profile of the white clover population 'Tienshan' under UV-B

This Chapter has been accepted for publication in *Annals of Botany*.

Abstract

This study was aimed at quantifying and identifying flavonoids involved in the response of nine populations of white clover (*Trifolium repens* L.) to ultraviolet-B radiation (UV-B). Plants were grown for 12 weeks in controlled environment rooms with or without supplemental UV-B radiation of 13.3 kJ m^{-2} d^{-1}. Methanol-water extractable flavonoids were quantified using high performance liquid chromatography (HPLC). Two major peaks showed significant enhancement in the HPLC chromatogram in response to supplemental UV-B. The structures of the compounds responsible were identified by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy (NMR) to be the flavonols quercetin-3-O-β-D-xylopyranosyl-(1→2)-β-D-galactopyranoside and kaempferol-3-O-β-D-xylopyranosyl-(1→2)-β-D-galactopyranoside. With supplemental UV-B, quercetin glycoside levels increased on average by 200% while the kaempferol glycoside response was much lower. Significant differences in flavonol accumulation were found among *T. repens* populations, both constitutively and in response to UV-B. Stress-adapted populations displayed particularly high flavonol levels under UV-B. There was an inverse correlation between plant productivity and quercetin accumulation. Furthermore, higher quercetin accumulation under UV-B was correlated with tolerance against UV-B-induced growth reduction. In conclusion, within-species comparisons in *T. repens* lend support to a distinct role for *ortho*-dihydroxylated flavonoids in the adaptation to UV-B stress and suggest particular advantages in the UV-B-induced biochemical adaptation for populations characterised by low habitat and plant productivity.

Introduction

Continued depletion of the stratospheric ozone layer has led to increased levels of ultraviolet-B radiation (UV-B, 290-315 nm) in the troposphere (McKenzie *et al.*, 1999). New Zealand is experiencing tropospheric UV-B doses which are up to 50% higher than at comparable northern hemisphere latitudes (Seckmeyer *et al.*, 1995) and which in summer can reach intensities similar to those at the tropics (McKenzie *et al.*, 1996). Increased UV-B levels have been implicated in a wide variety of plant responses. While earlier studies frequently focussed on damage caused by UV-B stress to particular plant species, UV-B is now also considered to be an important regulating factor at the ecosystem level (Caldwell *et al.*, 1998; Rozema *et al.*, 1997b). White clover (*Trifolium*

repens L.) is the dominant pasture legume in a number of temperate pasture ecosystem communities including most New Zealand swards where it performs vital functions due to its high feed value and as the major nitrogen-fixing plant species (Caradus *et al.*, 1996). Information on the UV-B response in *T. repens* is limited, however, especially at the population level and in regard to possible mechanisms of UV-B protection.

Several protective mechanisms against UV-B damage have been described for plants, ranging from repair functions (e.g. DNA repair, radical scavenging) (Barabas *et al.*, 1998; Britt, 1999) to preventative measures (e.g. UV-B screening, scattering and reflection) (Hoque and Remus, 1999). A particularly important role in this regard has been attributed to phenylpropanoids, including hydroxycinnamic acid derivatives and flavonoids with effective absorption in the UV-B spectral region (Hoque and Remus, 1999; Reuber *et al.*, 1996a; Sheahan, 1996). In addition to UV-screening, other important UV-B-protective properties ascribed to flavonoids include antioxidant activities (Dawar *et al.*, 1998) and energy dissipation via intramolecular proton transfer (Smith and Markham, 1998). Flavonoids can increase rapidly in response to UV-B radiation (Jordan, 1996) and are frequently found in or on epidermal layers where they have been shown to increase markedly following UV-B treatment (Reuber *et al.*, 1996b). Studies with mutants further highlight the importance of flavonoids for UV-B tolerance (Lois and Buchanan, 1994; Reuber *et al.*, 1996a).

Recent reports show that highly specific differential UV-B responses between closely related flavonoids are well conserved in the plant kingdom. Such differential responses were demonstrated in a liverwort (Markham *et al.*, 1998a), gymnosperms (Fischbach *et al.*, 1999; Schnitzler *et al.*, 1997), monocotyledons (Liu *et al.*, 1995; Markham *et al.*, 1998b; Reuber *et al.*, 1996a), as well as several dicotyledons, both herbaceous (Olsson *et al.*, 1998; Ryan *et al.*, 1998; Wilson *et al.*, 1998) and trees (Lavola, 1998). Several of these reports indicate a shift from B-ring monohydroxylated flavonoids towards their *ortho*-dihydroxylated equivalents under UV-B. The dihydroxylated flavonoids are seen to confer additional UV-B protection, which could, for instance, be mediated by higher relative antioxidant capacity (Cooper-Driver and Bhattacharya, 1998; Montesinos *et al.*, 1995).

Population differences in the biochemical and physiological stress response to UV-B within species have been reported in several studies (Correia *et al.*, 1999a; Sato and Kumagai, 1997; Ziska *et al.*, 1992). Two of the *T. repens* populations used in the present study, the cultivar 'Huia' and the Chinese ecotype 'Tienshan', have displayed population-specific differences in the secondary metabolic process of cyanogenesis under UV-B irradiation (see Chapter 7, Table 7-1). A developing body of recent evidence also points to population-dependent differences in specific flavonoid responses to UV-B (Lavola, 1998; Markham *et al.*, 1998b; Olsson *et al.*, 1998), suggesting an increasing need to examine the frequency and consistency of such effects within plant species. This has particular relevance to ecological theory of stress tolerance, predicting differential degrees of biochemical stress response depending on the productivity of plant species or populations and of their habitat (Diaz *et al.*, 1999; Grime, 1979; Poorter and Garnier, 1999). The present study examined the specific flavonoid UV-B response for nine populations of *T. repens* with diverse genetic and geographic backgrounds, including three ecotypes from natural environments, four cultivars and two breeding lines (see Table 3-1 in Chapter 3).

The immediate aim was to identify specific compounds involved in a potential flavonoid response after long-term UV-B irradiation for these nine populations. Considering the variety of backgrounds of the *T. repens* populations it was hypothesised that they would differ in the degree of flavonoid accumulation both within and between treatments. A further rationale in including several different populations was to examine whether links could be established between UV-B-related flavonoid accumulation and aspects of biomass production.

Materials and methods

Experimental design, plant material and cultivation, UV-B irradiation and harvest of biomass production have been described in Chapter 3. All attributes reported here were investigated in well-watered plants. Methods of quantitative flavonoid analysis were identical to those in Chapter 5. The General Analysis of Variance procedure in GENSTAT (Genstat, 1993) was used for analysis of main and interaction effects, while correlative studies were conducted with the GENSTAT Regression Analysis and Correlation procedures.

Flavonoid isolation and identification

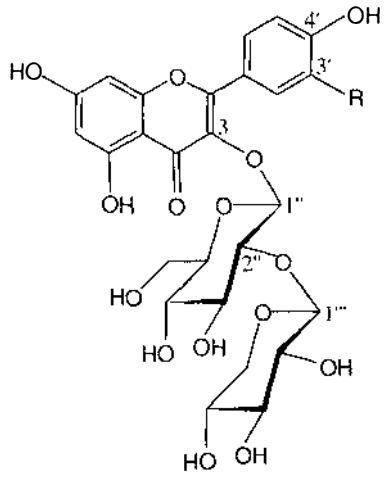
Dried and finely ground plant material was soaked for 24 h in MeOH:H_2O (1:1), containing 0.5% HOAc. The extract was filtered, concentrated under reduced pressure and vacuum-dried. The residue was chromatographed on a polyamide column using MeOH:H_2O (1:9 increasing to 9:1) to give six fractions. Fraction 6 was subjected to further column chromatography on cellulose using HOAc:H_2O (1:7). The fractions containing the two main 3-O-β-D-xylopyranosyl-(1→2)-β-D-galactopyranosyl derivatives of quercetin and kaempferol were pooled, concentrated and final purification was achieved by chromatography on a Merck LiChroprep RP-8 reverse phase Lobar column using CH_3CN:HOAc:H_2O (1.0:0.1:7). Acid hydrolysis was performed in MeOH-2N HCl (1:1) according to standard procedures (Markham, 1982). All 1D and 2D NMR spectra were recorded on a Bruker Avance spectrometer operating at 300.13 MHz for ^{1}H and 75.47 MHz for ^{13}C. Samples for NMR analysis were dissolved in DMSO-d_6 and run at room temperature.

Results

Flavonoid identification

HPLC analysis demonstrated that the major flavonoids present in the *T. repens* leaf samples were derivatives of the flavonols quercetin and kaempferol. A similar HPLC pattern in the nine populations showed that differences in flavonol accumulation between UV-B treatments were quantitative rather than qualitative. Two distinct peaks

increased notably under UV-B (Fig. 6-1). The flavonols **1** and **2** respectively responsible for these peaks were isolated and purified by polyamide, cellulose and reverse-phase RP-8 Lobar column chromatography, and identified by 1D (^{1}H, ^{13}C, ^{13}C-DEPT) and 2D (^{1}H-^{1}H COSY, ^{1}H-^{13}C TOCSY, HMBC) NMR spectroscopy.



1 R = OH
2 R = H

^{13}C and ^{1}H NMR data for **1** and **2** confirmed the quercetin and kaempferol skeletons, respectively. Acid hydrolysis of both **1** and **2** gave the sugars galactose and xylose. The presence of a galactopyranose and xylopyranose unit was confirmed by a ^{1}H-^{13}C TOCSY (total correlation spectroscopy) experiment which afforded the carbon subspectrum of each sugar residue. The ^{1}H NMR coupling constants of the anomeric protons indicated a β-configuration for the xylosyl and galactosyl units. The HMBC (heteronuclear multiple-bond correlation) spectrum showed the linkage of the galactosyl unit to the aglycone C-3 of **1** and **2**, while the xylose sugar was confirmed to be linked to C-2 of the galactose sugar. The flavonols **1** and **2** were thus identified as quercetin-3-O-β-D-xylopyranosyl-(1→2)-β-D-galactopyranoside and kaempferol-3-O-β-D-xylopyranosyl-(1→2)-β-D-galactopyranoside, respectively. These have previously been identified in horseradish, *Armoracia rusticana* (Larsen *et al.*, 1982).

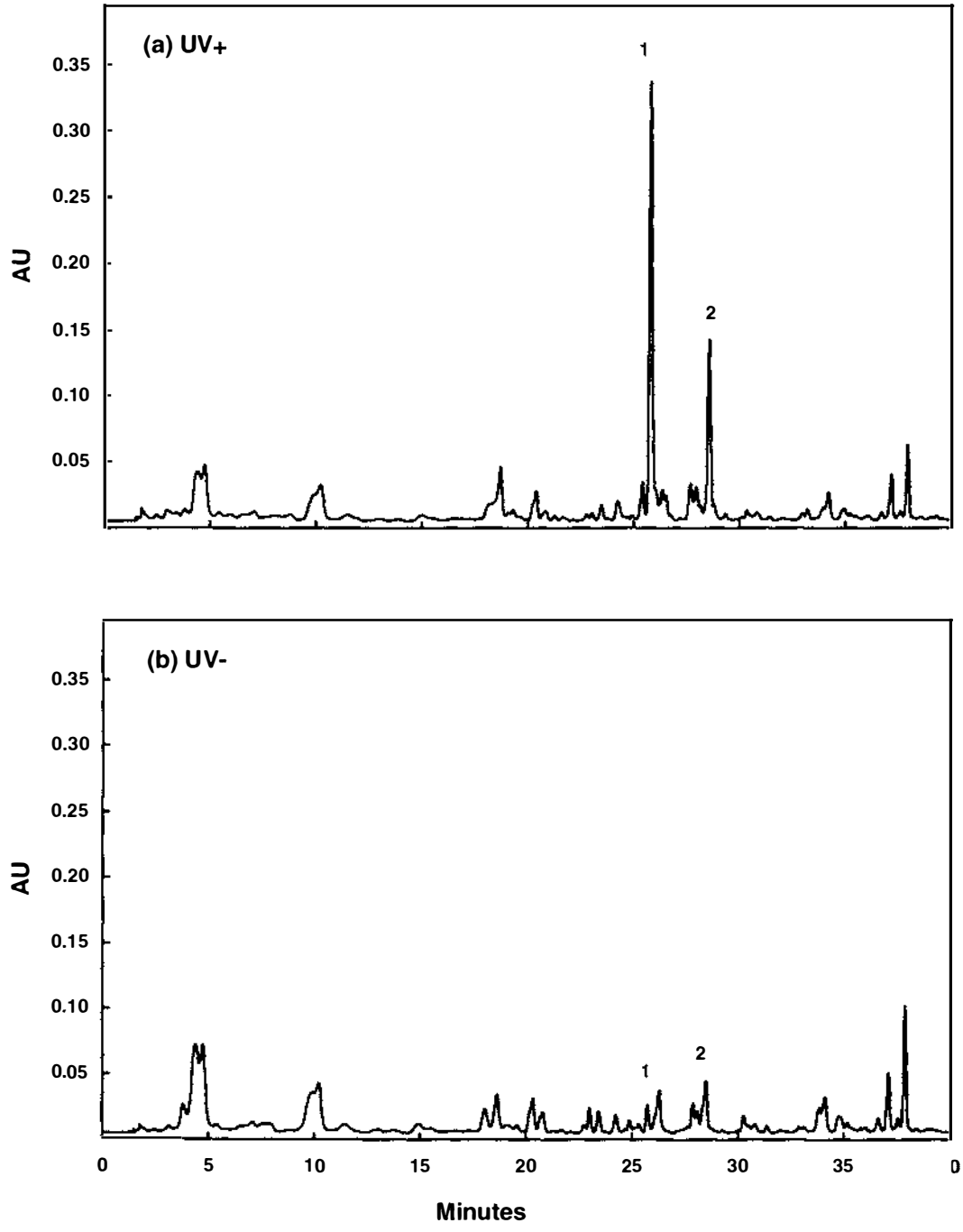


Fig. 6-1. Typical HPLC profiles of the *Trifolium repens* population 'Tienshan' grown with (a) and without (b) UV-B supplementation at a dose of 13.3 kJ m^{-2} day^{-1}. AU = absorbance units.

Total flavonols

This study revealed increases in flavonol levels for all *T. repens* populations exposed to UV-B treatment ($P < 0.001$). For many populations the rise in leaf flavonols exceeded 100% (Fig. 6-2). The populations differed significantly ($P < 0.001$) in leaf flavonol concentration. The ecotypes 'Häggås' and 'Tienshan' and cultivar 'Haifa' displayed the highest constitutive levels. 'Häggås' and 'Tienshan' contained more than three times the flavonol levels of 'Octoploid' (Fig. 6-2). UV-B-induced flavonol accumulation also differed significantly among populations ($P < 0.05$). 'Tienshan' displayed strongly increased flavonol levels and contained the highest UV-B-induced amounts among all populations (Fig. 6-2). The other two ecotypes ('Häggås' and 'Sarikamis') and the cultivar 'Huia' showed similar features. Together with most other cultivars and 'Octoploid', 'Syrian' displayed the lowest overall flavonol amounts under UV-B, about one third those of 'Tienshan' (Fig. 6-2). 'Syrian', together with the cultivar 'Haifa' also displayed the smallest, but still significant, flavonol increases.

Specific flavonols

Similar to total flavonols, quercetin glycoside levels consistently increased in all *T. repens* populations under UV-B ($P < 0.001$) (Fig. 6-3). On average, the UV-B-treated populations contained three times more quercetin glycosides than the controls. In contrast to this, the overall UV-B effect on kaempferol glycoside levels was only about half as pronounced and one third of the populations exhibited no significant enhancement (Fig. 6-3). In non-irradiated plants, the concentration of specific flavonols (Fig. 6-3) differed more extensively among the *T. repens* populations than that of total flavonols ($P < 0.001$). Quercetin glycoside accumulation was notably low in the cultivar 'Kopu' (only 5% of the average levels) and 'Octoploid' (20% of average), while the amount in the ecotypes 'Häggås' and 'Tienshan' was about double to most other populations. Population differences for constitutive kaempferol glycoside accumulation ranged from cultivar 'Haifa' (approximately twice the average) to another cultivar, 'Prestige', with only 25% of average levels (Fig. 6-3).

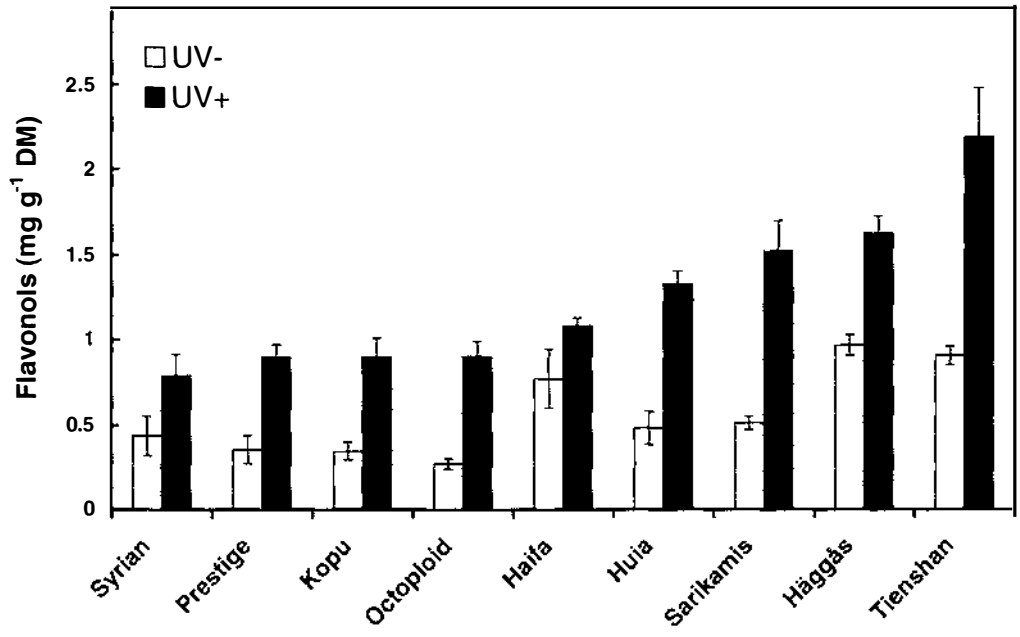


Fig. 6-2. Total flavonol concentration in the leaves of nine *Trifolium repens* populations grown with and without UV-B supplementation of 13.3 kJ m^{-2} day^{-1}. The bars are the standard errors of the means. DM = dry matter.

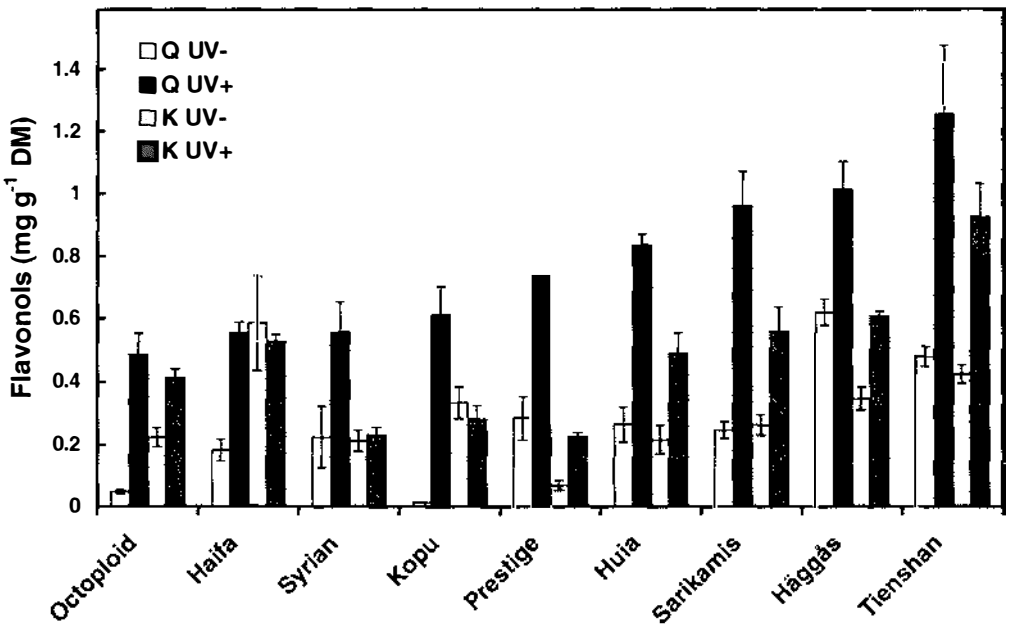


Fig. 6-3. Quercetin and kaempferol concentration in the leaves of nine *Trifolium repens* populations grown with and without UV-B supplementation of 13.3 kJ m^{-2} day^{-1}. The bars are the standard errors of the means. DM = dry matter.

Population differences were also maintained under UV-B ($P < 0.001$) (Fig. 6-3). High concentrations of both flavonol derivatives and pronounced UV-B-induced increases were found in the ecotypes, particularly 'Tienshan', reflecting the observations made for total flavonols. Similarly marked responses were observed for the quercetin and kaempferol glycosides in 'Huia'. Compared to the ecotypes, quercetin levels under UV-B were about half or less in 'Octoploid' and 'Haifa', while kaempferol glycoside levels were more similar (Fig. 6-3). 'Haifa', together with 'Kopu', displayed no measurable increase in kaempferol glycosides under UV-B. 'Kopu', however, displayed a pronounced increase in quercetin derivatives under UV-B. In the populations 'Prestige' and 'Syrian' the low constitutive kaempferol glycoside levels also remained minimal under UV-B at about one third or less of the ecotypes. Furthermore, there was a significant relationship between constitutive and UV-B-induced levels for quercetin ($r = 0.785$, $P < 0.05$), but not for kaempferol glycosides.

Q:K ratio

Significant increases ($P < 0.001$) in the quercetin:kaempferol glycoside (Q:K) ratio for most populations further illustrate the tendency for UV-B-generated quercetin accumulation, rather than kaempferol (Fig. 6-4). Low specific constitutive flavonol levels strongly affected Q:K ratios. Especially low constitutive Q:K ratios were found in the quercetin-poor populations 'Kopu' and 'Octoploid'. The population lowest in kaempferol glycosides, cultivar 'Prestige', displayed the highest Q:K value. Between these extremes there was a significant trend towards higher relative constitutive quercetin glycoside amounts in the *T. repens* ecotypes and cultivar 'Huia' ($P < 0.001$). In contrast to the constitutive Q:K ratios, UV-B-irradiated *T. repens* populations consistently contained more quercetin than kaempferol glycosides (Q:K > 1), ($P < 0.001$) (Fig. 6-4). UV-B-induced Q:K ratios were again highest in the cultivar 'Prestige', followed by 'Syrian' and 'Kopu'. Q:K ratios were intermediate in the *T. repens* ecotypes and lowest in cultivar 'Haifa' and in 'Octoploid' (Q:K near 1).

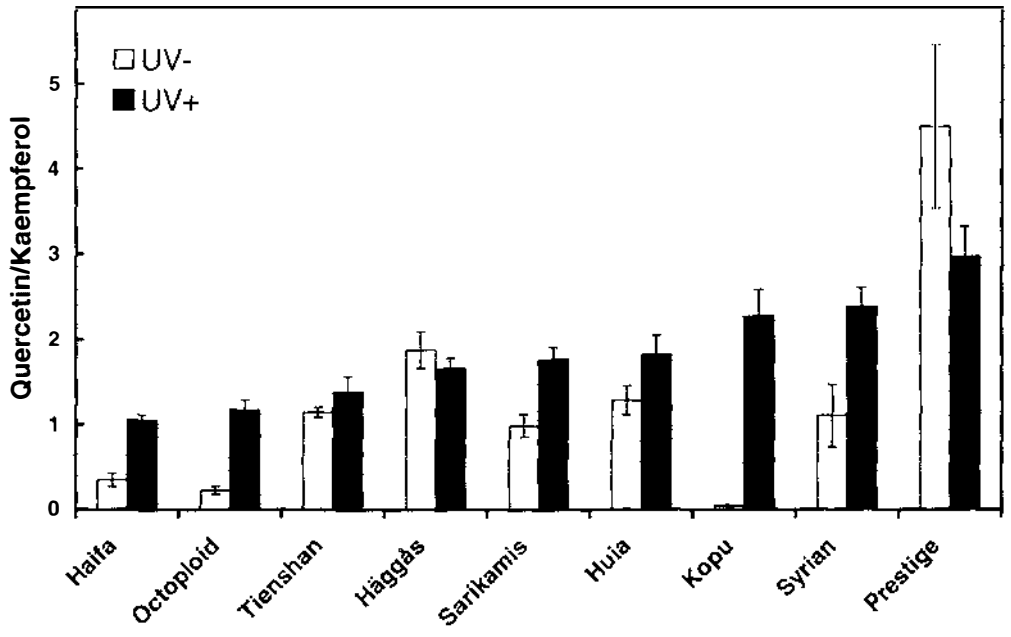


Fig. 6-4. Ratio of quercetin to kaempferol (Q:K) derivatives in nine *Trifolium repens* populations grown with and without UV-B supplementation of 13.3 kJ m^{-2} day^{-1}. The bars are the standard errors of the means.

In addition to significant differences among the *T. repens* populations in altering the Q:K ratio under UV-B, there was also an inverse relationship between such changes and constitutive Q:K ratios (r = -0.894, $P < 0.01$). 'Kopu', the population with the lowest initial Q:K ratio displayed the highest Q:K increase, while populations with notably more constitutive quercetin than kaempferol glycosides ('Häggås' and 'Prestige') displayed the opposite Q:K response. Similar to quercetin glycoside accumulation per unit leaf dry matter, the quercetin amounts relative to kaempferol also displayed a relationship between initial and UV-B-elicited levels ($r = 0.663$, $P = 0.05$).

Flavonol relationships to biomass production

Constitutive plant productivity and UV-B tolerance correlated with several aspects of quercetin - but not kaempferol - glycoside accumulation. There was a significant inverse correlation between productivity of the *T. repens* populations to constitutive quercetin glycoside levels (r = -0.866, $P < 0.01$) and Q:K ratios ($r = -0.692$, $P < 0.05$). Quercetin glycoside accumulation under UV-B was also inversely linked to constitutive

productivity (Fig. 6-5) and in turn tended to relate to UV-B tolerance of the *T. repens* populations (Fig. 6-6). Examination of the growth responses for the nine *T. repens* populations suggests significant UV-B tolerance for the lower-yielding ecotypes and sensitivity to UV-B for more productive cultivars ($P < 0.05$). The UV-B-mediated growth response also showed some relationship to increases in total flavonols (r = 0.639, $P = 0.064$) and quercetin glycosides (r = 0.595, $P = 0.091$).

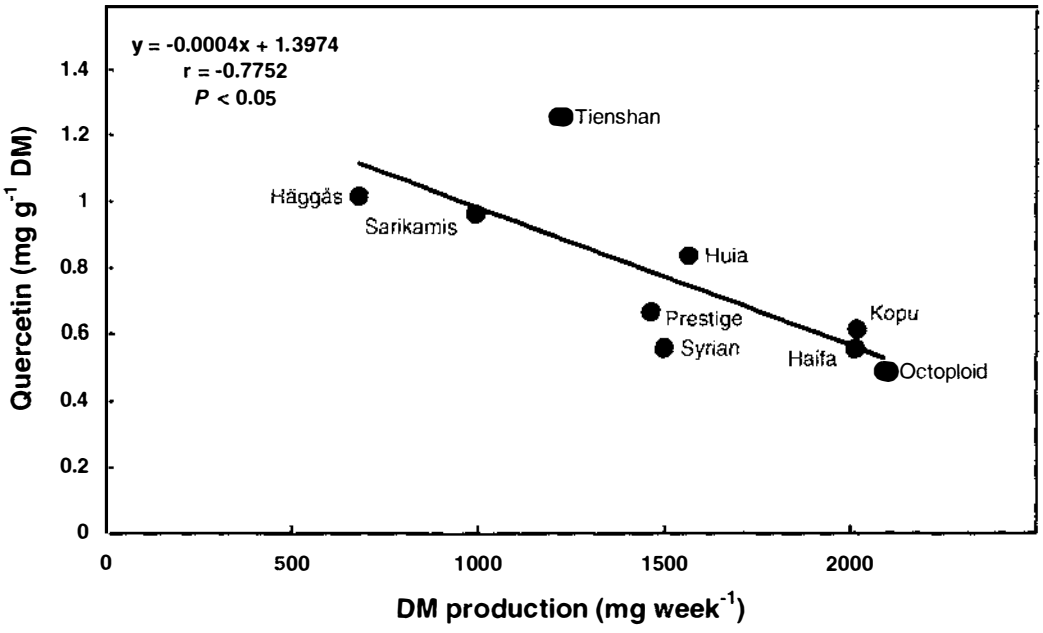


Fig. 6-5. Relationship between UV-B-induced quercetin accumulation and constitutive dry matter (DM) production for nine *Trifolium repens* populations.

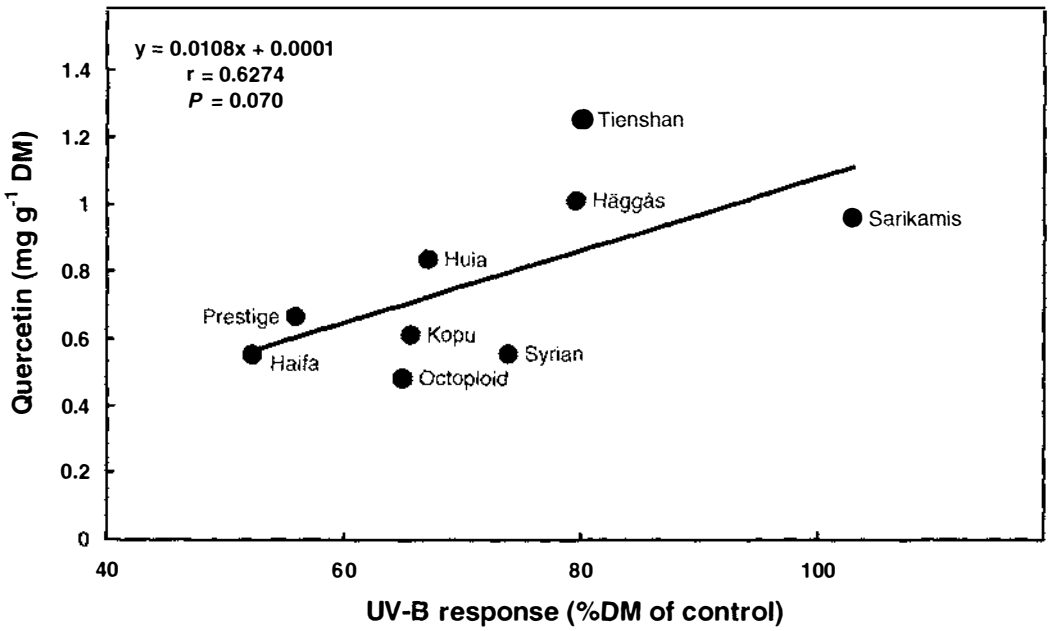


Fig. 6-6. Relationship between UV-B-induced quercetin accumulation and relative growth (dry matter production under UV-B expressed as a percentage of that of the control) in response to elevated UV-B for nine *Trifolium repens* populations. DM = dry matter.

Discussion

Total flavonols

This study investigated *Trifolium repens*, a species previously not described with respect to its flavonoid UV-B response. After 12 weeks of UV-B exposure in controlled environment conditions there was evidence for treatment-, population- and compound-specific differential accumulation of flavonols which are likely to contribute towards protection from UV-B radiation.

The involvement of flavonols in the UV-B response has been reported for several plant species, including other legumes such as soybeans, *Glycine max* (Middleton and Teramura, 1993). The levels of flavonols in *T. repens* and the pronounced UV-B-induced change in their amounts by more than 100% (Fig. 6-2) are similar to those observed elsewhere, for instance in *Brassica* (Olsson *et al.*, 1998) where total flavonols, composed of kaempferol and quercetin glycosides, exhibited up to 150% enhancement in response to UV-B. Similarly, the increment for flavonoid compounds (mainly the flavone saponarin) in *Hordeum vulgare* (barley) was about 100% (Liu *et al.*, 1995). Accumulation of flavonoids is a frequently observed response to UV-B radiation (Meijkamp *et al.*, 1999) and has been linked to UV-B absorption characteristics displayed by flavonoids (Hoque and Remus, 1999) as well as to the potential for these compounds to act as radical-scavenging antioxidants (Cooper-Driver and Bhattacharya, 1998). However, the antioxidant function of flavonoids is complex and depends on a variety of factors, including compartmentalisation, redox potential, presence of double bonds, glycosylation and hydroxylation (Bors *et al.*, 1995; Cooper-Driver and Bhattacharya, 1998; Rice Evans *et al.*, 1996). This complexity therefore also needs to be taken into account in the consideration of possible antioxidant functions for increased flavonoid levels under UV-B (Bornman *et al.*, 1997; Olsson *et al.*, 1998). Recent studies on keto-enol tautomerism in the ground- and excited states suggest a mechanism by which kaempferol and quercetin glycosides might provide protection against UV-B damage by (harmless) energy dissipation (Smith and Markham, 1998).

Differential UV-B effects on quercetin and kaempferol glycosides

There was a clear trend among the flavonols for UV-B-induced accumulation of quercetin glycosides (Fig. 6-3). On average, the *T. repens* populations double their Q:K ratio under UV-B, and it appears this increase is only limited for populations already high in intrinsic quercetin glycosides relative to kaempferol (Fig. 6-4). Studies in *Anethum graveolens* (dill) provided early indications towards selective formation of quercetin glycosides as a response to UV-B (Möhle *et al.*, 1985; Möhle and Wellmann, 1982). However, only recently an increasing number of studies have pointed at differential UV-B effects favouring B-ring-dihydroxylated flavonoids over their monohydroxylated counterparts, for instance in *Marchantia* (Markham *et al.*, 1998a), *Hordeum* (barley) (Liu *et al.*, 1995; Reuber *et al.*, 1996a), *Oryza* (rice) (Markham *et al.*, 1998b), *Petunia* (Ryan *et al.*, 1998) and *Brassica* (Olsson *et al.*, 1998). The present studies on *T. repens* not only confirm such differential flavonol effects under long-term UV-B treatment for a leguminous plant species, but also show that the attainment of a higher Q:K ratio under UV-B is well-conserved for several genetically diverse populations within the same species.

It is unlikely that preferential quercetin accumulation would lead to higher UV-B-absorption, compared to kaempferol (Lavola *et al.*, 1997; Mabry *et al.*, 1970). UV-B radiation generates free radicals (Hideg and Vass, 1996) and higher free radical antioxidant activity relative to their monohydroxylated equivalents has been reported for *ortho*-dihydroxylated flavonoids (Montesinos *et al.*, 1995). A possible additional relative advantage for quercetin compounds has also been indicated by photochemical studies (Smith and Markham, 1998). These suggest that quercetin glycosides may be better able to dissipate potentially harmful UV-B radiation through tautomerisation than the equivalent kaempferol glycosides. Specific quercetin accumulation has been observed as a response to a number of other forms of stress, ranging from heavy metal pollution (Loponen *et al.*, 1998) and nitrogen deficiency (Bongue Bartelsman and Phillips, 1995) to electron-donating paraquat application (Steger-Hartmann *et al.*, 1994). In combination with the findings presented here, this underscores a prominent role for *ortho*-dihydroxylated flavonol accumulation in the general biochemical plant strategy against environmental stress.

Population differences in the accumulation of total flavonols

UV-B-generated flavonoid accumulation differed markedly among the nine genetically diverse *T. repens* populations. There were important population-specific differences for the accumulation of quercetin, kaempferol and total flavonols and the capacity to increase these compounds under UV-B. This is in accordance with more general investigations reporting population-specific effects on UV-B-generated flavonoid accumulation (Dai *et al.*, 1992; Dumpert and Knacker, 1985; Murali and Teramura, 1984). The *T. repens* ecotypes stood out among the other populations in that they usually were highest in specific and total flavonols under both treatment conditions, and were also able to increase these levels most, compared to control conditions. Overall the best performing population was 'Tienshan' which had high constitutive and UV-B-induced total flavonols and also displayed pronounced UV-B-induced increases. This high performance was usually also seen in the other two ecotypes, 'Sarikamis' and 'Häggås'. The origin of 'Tienshan' and 'Sarikamis' at high altitudes could suggest adaptation to higher UV-B levels resulting in increased flavonol accumulation. While some reports indicate a relationship between natural UV-B background levels and the production of UV-B-attenuating pigments, including flavonoids (Rozema *et al.*, 1997a; Ziska *et al.*, 1992), other studies show no such pattern (Pilon *et al.*, 1999). Similarly, the Swedish ecotype 'Häggås', originating at high latitudes (Nordiska Genbanken, 1993), and thus representing a habitat with lower UV-B levels, displays higher flavonol accumulation. This suggests that no overall association exists between UV-B habitat and flavonol production in *T. repens*.

There are several other features shared by the *T. repens* ecotypes. In addition to lower productivity, another common denominator for the ecotypes is adaptation to low temperatures. Low-temperature stress can increase antioxidant capabilities due to enhanced phenylpropanoid accumulation, including flavonoid production (Grace *et al.*, 1998). Furthermore, these populations all derive from environments with low annual rainfall, at precipitation levels considered marginal for *T. repens* growth (Jones and Lowe, 1993). It can therefore be suggested that the *T. repens* ecotypes in this investigation are also particularly well adapted to extended periods of drought. Tolerance to other forms of stress such as drought has been related to higher UV-B tolerance (Al-Oudat *et al.*, 1998). More specifically, drought tolerance has been linked

to higher flavonoid content (Pizzi and Cameron, 1986) and could further explain the higher constitutive and UV-B-induced flavonoid levels for these ecotypes. The high flavonol-accumulating capabilities of the ecotypes were closely followed by the cultivar 'Huia', which displayed similarly high levels and increases. This general-purpose cultivar has been used extensively in temperate pasture ecosystems, and exhibits relatively high productivity and an ability to adapt to a variety of environments with high degree of plasticity (Caradus *et al.*, 1993b; Caradus and Woodfield, 1997). It appears that biochemical adaptation in 'Huia' was characterised by elevated flavonol production under UV-B during the 12 week growth period.

The remaining populations with low to intermediate flavonol accumulation generally represented the higher-yielding cultivars and breeding lines. This included the cultivars 'Haifa' and 'Kopu' which are also sensitive to other forms of stress such as drought (Macfarlane *et al.*, 1990) and frost (Caradus *et al.*, 1989a). Flavonol accumulation in 'Prestige' and 'Syrian' could be related to selection for specific stress tolerance. Low kaempferol but high quercetin accumulation in 'Prestige' could be due to some drought adaptation (Caradus and Woodfield, 1997). There could also be flavonol-independent defence mechanisms in 'Syrian' which has been selected for leaf rust tolerance (Caradus *et al.*, 1990a). Flavonol accumulation was also low in the highly productive population 'Octoploid'.

The general picture emerging from these observations is that predominant carbon flow in favour of biomass accumulation could come at some cost for sequestration towards other carbon-requiring processes, such as production of specific flavonoids. There seem to be general advantages for populations from natural environments not selected or bred for productivity but adapted to a variety of stress forms. An explanation for this can be found in ecological models of stress tolerance, proposing distinct plant types depending on primary plant strategies (Grime, 1979; Poorter and Garnier, 1999). Such models of plant specialisation predict that less productive populations originating in stress-prone habitats would display a higher degree of cellular biochemical stress acclimation, compared to more morphology-based stress responses expected in populations largely selected and bred for high productivity (Grime and Campbell, 1991). Thus the lower plant yield and reduced habitat productivity of the *T. repens* ecotypes could place them

at a particular advantage in regard to the accumulation of specific compounds required in the protection against increased UV-B irradiation.

Population differences in the accumulation of quercetin and kaempferol

In this study, an inverse relationship was observed between plant productivity and UV-B-generated accumulation of quercetin (Fig. 6-5), but not kaempferol glycosides. A limited number of published studies have reported population differences in specific flavonoid responses. Differential increases have been documented for quercetin glycosides, but also for myricetin and for kaempferol glycosides in several birch populations (Lavola, 1998). Furthermore, population-specific efficiency in UV-B-generated *ortho*-dihydroxylated flavonoid accumulation, relative to their monohydroxylated counterparts, has been observed for two cultivars in rice (Markham *et al.*, 1998b) and in *Brassica* (Olsson *et al.*, 1998). The present study established differential effects of UV-B and increases in flavonol glycosides for an extended range of populations of the leguminous species *T. repens*. The degree of specific flavonol accumulation in the natural, unselected populations and Q:K ratios between 1 and 2 under both treatment conditions show that they can maintain efficiency in the accumulation of quercetin relative to kaempferol during the accumulation of high flavonol amounts under UV-B. Similar properties could again be observed in the general-purpose cultivar 'Huia'.

However, there were differences in these features among the remaining, more specialised and selected populations, accumulating lower amounts of flavonols. The maximal Q:K ratios and minimal kaempferol levels for 'Prestige', 'Syrian' and 'Kopu' under UV-B indicate that these populations are to some degree able to compensate for the relatively low available flavonol levels with more efficient quercetin glycoside accumulation. While this could be related to selection for specific stress tolerance in 'Syrian' and 'Prestige', it could for 'Kopu' be indicative of the high plasticity it shares with its 3/8-progenitor 'Huia'. Low constitutive quercetin glycoside accumulation similar to 'Kopu' and strong increases in these levels and in the Q:K ratio under UV-B have been reported in *Brassica* (Olsson *et al.*, 1998). Particularly low quercetin glycoside levels were found in the highly productive populations 'Octoploid' and 'Haifa', which - in addition to low constitutive Q:K levels - also had minimal Q:K ratios under

UV-B. It thus appears that in addition to limited flavonol availability these two populations are further disadvantaged in their quercetin production due to low efficiency of UV-B-generated quercetin glycoside accumulation, relative to kaempferol. Taken together these findings suggest a central role for the dihydroxylated:monohydroxylated flavonoid ratio especially in circumstances of limited flavonoid accumulation. This conclusion can also be drawn from similar observations in other studies (Markham *et al.*, 1998a; Ryan *et al.*, 1998).

Relationship of flavonol levels to UV-B tolerance

In addition to an inverse relationship between quercetin levels and plant productivity (Fig. 6-5), UV-B-sensitivity in the *T. repens* populations also appears to be linked to UV-B-induced accumulation of quercetin, rather than kaempferol glycosides (Fig. 6-6). Other reports also link higher B-ring hydroxylation to UV-B tolerance, for instance in rice (Markham *et al.*, 1998b) and *Petunia* (Ryan *et al.*, 1998). In addition, indirect advantages were noted for high quercetin-containing populations such as the *T. repens* ecotypes 'Tienshan', 'Sarikamis' and 'Häggås'. *T. repens* populations with an ability to accumulate high levels of constitutive quercetin glycosides also displayed higher UV-B-induced quercetin concentrations. This also suggests a constitutive advantage for quercetin-rich populations, as high final quercetin amounts showed correlation with UV-B tolerance (Fig. 6-6). No such relationships could be observed for kaempferol accumulation. Stress-induced stimulation of specific phenylpropanoid branch pathways has been documented (Dixon and Paiva, 1995) and the present findings lend support to views attributing quercetin a specific role in stress acclimation (Olsson *et al.*, 1998). Additional trends observed here - linking UV-B growth response with the capacity to change flavonoid levels - are consistent with other reports of a general advantage for plants able to increase flavonoid levels under UV-B (Lois, 1994; Murali and Teramura, 1984; Olsson *et al.*, 1998).

Conclusions

This study demonstrated flavonoid-specific responses to long-term UV-B irradiation with particular advantages for quercetin glycoside accumulation. Population comparisons show marked intraspecific differences in leaf flavonol accumulation and support the concept that the capacity to specifically accumulate higher levels of quercetin glycosides under UV-B confers a higher degree of UV-B tolerance. The linkage between (1) relatively high flavonol (and more specifically quercetin) accumulation, (2) stress-adapted populations of lower constitutive plant productivity, and (3) UV-B tolerance is in accord with current ecological theory on stress tolerance. Stress-adapted ecotypic populations appear particularly advantaged while the inverse correlation between plant productivity and quercetin levels as well as UV-B sensitivity clearly points at disadvantages for the most productive *T. repens* populations. These populations have largely been selected and bred for high productivity and it is possible that this affects other attributes such as the capacity to accumulate specific secondary compounds.

An increase in dihydroxylated flavonoid compounds is a well-conserved UV-B response for markedly different plant species. The present study demonstrates that this observation also applies to distinctly different populations within a species. These findings, in conjunction with similar observations in studies of other forms of plant stress further suggest a particular role for *ortho*-dihydroxylated flavonol glycosides in the biochemical plant strategy as a response to stress. For future UV-B studies this highlights a need for investigations of the chain of events from UV-B treatment via specific flavonol accumulation to possible protective functions. The present findings suggest a requirement to include a wider range of populations in such studies as the quality and quantity of the UV-B response could differ considerably within a species. Field testing could provide further confirmation of the role of *ortho*-dihydroxylated flavonoids as potential predictors for UV-B sensitivity.

Chapter 7. Differences in *Trifolium repens* L. responses to ultraviolet-B radiation: foliar chemistry and consequences for two lepidopteran herbivores

Bioassay studies with lepidopteran larvae

This Chapter has been accepted for publication in *Oecologia*.

Abstract

White clover growing in New Zealand is experiencing increasing levels of ultraviolet-B (UV-B) radiation as a result of ozone depletion. The study evaluated the effects of UV-B radiation on foliar chemistry of two populations of white clover (*Trifolium repens* L.), 'Huia' and 'Tienshan', and consequences for the performance of armyworms (*Spodoptera litura*) and cutworms (*Graphania mutans*). Plants were grown in controlled environment rooms with and without supplemental UV-B radiation at a dose of 13.3 kJ m^{-2} d^{-1}, corresponding to a 25% mid-summer ozone depletion above Palmerston North, New Zealand. In both white clover populations UV-B radiation elicited changes in foliar chemistry, including slight increases in nitrogen concentrations and decreases in carbohydrate concentrations. In addition, the population 'Huia' showed decreases in fibre concentrations and marked increases in cyanogenic activity. No change in UV-absorbing compounds was detected in either population. Long- and short-term feeding trials were conducted to assess dietary effects on insect growth, consumption and food utilisation. Changes in the performance of both insect species were generally small. The most pronounced effect was a 36% reduction in weight of *S. litura* after two weeks of feeding on 'Huia' grown at high UV, but larval development times were only slightly prolonged and pupal weights were unaffected. *S. litura* short-term performance was affected by differences in white clover population. Long-term performance of *G. mutans* was not affected and short-term performance (stadium duration and consumption rate) was only marginally affected by the high UV treatment. It is concluded that the effects of elevated UV-B radiation on white clover plant chemistry can be specific to certain plant populations. The differences in sensitivity of the two generalist insect species suggest that effects may also be specific to certain plant-herbivore associations. These results indicate that future UV-B herbivory studies should examine genotypic effects in both plants and animals.

Introduction

Recent changes in UV-B radiation due to atmospheric ozone depletion are widely recognised as having significant consequences for plant biochemistry, physiology, and productivity (Caldwell *et al.*, 1998). UV-B-mediated changes in the quantity and quality of plant components are also likely to affect heterotrophs, via trophic interactions and

cascades (Mazza *et al.*, 1999). Exceedingly few studies, however, have addressed the impact of UV-B on such interactions (Paul *et al.*, 1997).

The consequences of high UV-B irradiances for consumer organisms are of particular interest in New Zealand, where UV-B doses are markedly higher than at comparable latitudes in the northern hemisphere (Seckmeyer and McKenzie, 1992). This is due to hemispheric differences in stratospheric and tropospheric (pollutant) ozone, differential aerosol loading, and because the distance between the Sun and Earth is minimal during the Austral summer (Seckmeyer and McKenzie, 1992). Furthermore, while studies have demonstrated UV-B sensitivity for several legumes (e.g. Krupa *et al.*, 1998b; Teramura, 1990) only limited research has been conducted on UV-B sensitivity of white clover (*Trifolium repens* L.) (Matthew *et al.*, 1996; Rozema *et al.*, 1997a). White clover is the dominant legume of the pasture ecosystems in New Zealand, performing a vital role for ecosystem processes as a primary source of soil nitrogen and also as a highly nutritious component of the diet for grazing ruminants (Caradus *et al.*, 1996).

The study investigated the impact of UV-B radiation on the chemical composition of two populations of white clover, 'Huia' and 'Tienshan', and subsequent effects on the performance of two phytophagous insects, *Spodoptera litura* (Lepidoptera: Noctuidae) and *Graphania mutans* (Lepidoptera: Noctuidae). The white clover population 'Huia' is a commonly used New Zealand cultivar, whereas the 'Tienshan' population is a high-altitude ecotype from northwest China. The generalist tropical armyworm, *S. litura*, became established in northern New Zealand in the mid-1970s. It has been known to cause severe, though localised, defoliation of clover (Chapman, 1984). The generalist cutworm, *G. mutans*, is native to New Zealand and causes damage to pasture, vegetable and fruit crops (McGee, 1987; McGregor *et al.*, 1987).

A small but growing number of studies have assessed the effects of UV-B-mediated changes in plant chemistry on insects. Some studies suggest that UV-B-induced effects may alter herbivory through changes in plant primary metabolism, e.g. increased nitrogen levels (Hatcher and Paul, 1994) and decreased sugar concentrations (Yazawa *et al.*, 1992). Other research points to the involvement of secondary metabolites such as flavonoids and related compounds of the shikimic acid pathway, (e.g. Grant-Petersson and Renwick, 1996).

Plant chemistry linked to primary metabolism and of relevance to insect feeding was analysed. The study also examined aspects of plant secondary metabolism, including levels of UV-absorbing compounds and cyanogenesis, a mechanism present in several populations of white clover and more than 2000 other angiosperm species (Kakes, 1990). Hydrogen cyanide (HCN) production eventuates when cyanide-containing glucosides (linamarin and lotaustralin) and the hydrolytic β-glucosidase linamarase, normally separated by compartmentalisation, are brought together during lesion of plant tissues. White clover is known to show increases in cyanogenesis as a common response to several forms of stress, including herbivory, frost and drought (Caradus *et al.*, 1990b; Williams, 1987a). UV-B could possibly alter this potential defence pathway by a general stimulatory effect on secondary metabolism (Rozema *et al.*, 1997b).

In this study, it was sought to determine whether UV-B would affect key attributes of white clover chemical composition, and whether the two white clover populations would be differentially affected. It was also predicted that performance of *S. litura* and *G. mutans* would decline when reared on foliage of white clover grown under high levels of UV-B, and that the magnitude of this effect would vary between white clover populations.

Materials and methods

Experimental design

The overall design was a 2 x 2 factorial, with two levels of UV-B radiation and two populations of white clover. Trolleys containing the plant material were rotated regularly within and between the two experimental rooms in order to equalise potential location effects. In a separate room maintained with no UV supplementation, feeding experiments were first conducted with *S. litura*, then repeated with *G. mutans*.

Plant cultivation and UV irradiation

Stolon cuttings of 'Huia' and 'Tienshan' white clover populations were planted into separate 3 litre pots containing 3 kg of soil (Karapoti brown sandy loam). The soil was

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 by an automatic drip irrigation system. Relative humidity in the growth rooms was 70% and day/night temperatures were 24°C and 18°C, respectively. Daylength was 14 hours and mean photosynthetic photon flux (PPF) 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. PPF was measured using a LI-COR 185 meter with a LI-190S quantum sensor.

Elevated UV-B radiation was supplied by Philips TL 40W/12 RS fluorescent UV tubes, enclosed in cellulose diacetate filters. Biologically effective UV-B levels were 13.3 kJ m^{-2} d^{-1}, calculated from the generalised plant action spectrum (Caldwell, 1971) and normalised to 300 nm. The UV treatment started 1 h after onset of the light period and ended 1 h before darkness. A feedback control system continuously monitored and adjusted the output of the UV lamps in response to degradation of the filters and ageing of the tubes in order to maintain the set UV-B level. The system incorporated a Solar Light Company UV-B biometer to measure incident radiation. This sensor was regularly calibrated against the generalised plant action spectrum (Caldwell, 1971) using a Bentham DM150 UV spectroradiometer. In the control room, Mylar filter sheets were used to screen out UV-B but maintain UV-A levels similar to those in the UV-B treatment. The plants were subjected to the UV treatments for 29 days prior to initiation of insect bioassays.

Sampling procedure for plant nutrient analyses

Leaf samples for chemical analyses were collected from five pairs of pots within each treatment. Fully open trifoliate laminae were frozen in liquid nitrogen and freeze-dried. Samples were then finely ground in a coffee grinder and stored at -20 °C until chemically analysed.

Nitrogen

Samples containing 10 mg freeze-dried material were automatically loaded into a Carlo-Erba Instruments NA 1500 Series II Analyser to determine nitrogen levels (Carlo-Erba, 1988). The instrument detects N_2 by thermal conductivity after flash combustion

(around 1000°C) of the plant material. The resulting electrical signal is then converted to % N_2 present in the sample using internal calibration standards (atropine, phenantrene).

Available carbohydrates

Available carbohydrates (AC) in the white clover samples were determined as reducing sugars using the *p*-hydroxybenzoic acid hydrazide (PAHBAH) procedure, yielding soluble carbohydrates and starch (Blakeney and Mutton, 1980; Southgate, 1991). Freeze-dried samples (100 mg) were solubilised with 0.4 ml dimethyl sulfoxide (DMSO). After boiling in DMSO for 30 min, the carbohydrates in the solution were exposed to enzyme digestion, converting polysaccharides into simpler sugars. 1.6 ml amylase solution (Southgate, 1991) was added and the solution boiled for another 15 min. This was followed by 30 min incubation (50°C) in 100 µl amyloglucosidase solution (Southgate, 1991). A glucose standard solution (5 mg ml^{-1}) was prepared and assayed alongside the white clover sample batches to determine the percentage of AC in the freeze-dried material. The sample digests were prepared for colorimetric determination using standard procedures (Blakeney and Mutton, 1980), using double precipitation in 400 µl barium hydroxide and 100 µl zinc sulphate solution, followed by precipitation for 30 min in 7.5 ml cold ethanol (absolute). After centrifugation, 100 µl of the supernatant was subjected to acid hydrolysis (200 µl of 0.5 M HCl) in a boiling water bath for 30 min. This was followed by addition of 2 ml PAHBAH solution (0.5 g 100 ml^{-1}), vortex mixing and further boiling for 6 min. The tubes were then cooled and 4 ml water was added. Absorbance of the solution was measured as reducing sugars at 420 nm (Blakeney and Mutton, 1980).

Neutral detergent fibre

Fibre content was measured with the neutral detergent fibre (NDF) procedure (Southgate, 1991; van Soest *et al.*, 1991). Samples of 100 mg freeze-dried material were boiled in 10 ml neutral detergent solution (Southgate, 1991), centrifuged at 2000 *g* and the residue digested at 37°C for 18 h with 5 ml enzyme solution (2.5% α-amylase [w/v] in 0.1 M phosphate buffer, pH 7) (van Soest *et al.*, 1991). These procedures remove

pectins and plant cell contents (proteins, sugars and lipids), leaving a fibrous residue (a NDF), consisting primarily of plant cell wall components (hemicellulose, cellulose and lignin). Centrifugation was followed by several washing steps of the residue in water (10 ml), followed by washing in 96% ethanol and lastly in acetone. Following evaporation and desiccation, the resulting final mass of the residue corresponds to the fibre content (%NDF) in the sample (Southgate, 1991).

Cyanogenesis

Cyanide (HCN) production of fully unfolded young trifoliate laminae was examined in a two-step procedure using a modified picrate paper test (Williams *et al.*, 1998). The picrate paper technique is a semi-quantitative method involving scoring of colour intensity by eye, which gives satisfactory correlation with quantitative procedures (Melville *et al.*, 1940). In the first assay, a leaflet from each pot was tested for HCN production with picric acid as indicator. After addition of 10 µl toluene and overnight incubation at 37°C, the colour change in picric acid was examined by comparison with a standard colour scale, ranging from 0 (unchanged, yellow) to 5 (dark orange) (Corkill, 1940). Cyanogenesis intensity per leaf was expressed on a dry weight basis. Plants that did not prove cyanogenic in this first test were then examined in a second assay for the presence of substrates (linamarin and lotaustralin). The same picric acid procedure was used, but included addition of 20 µl of 0.5% linamarase (a glucosidase enzyme) to liberate HCN (Williams *et al.*, 1998). This amount was used so as to assume that enzyme availability did not limit the reaction.

UV-absorbing compounds

Standard protocols were used to estimate levels of methanol-extractable UV-absorbing compounds (Mirecki and Teramura, 1984). For each sample, four fully unfolded young trifoliate laminae were oven-dried and 15-20 mg of ground material was weighed into Eppendorf tubes. The extraction was performed with 1.2 ml MeOH:H_2O:HCl (79:20:1), followed by spectrophotometric analysis. Absorbance readings at 300 nm were calculated on the basis of leaf dry weight. This procedure was repeated on seven replicates for each white clover population under each UV-B treatment.

Spodoptera litura *bioassays*

S. litura egg masses were provided by Biodiscovery of Auckland, New Zealand. All rearing was conducted in a controlled environment room similar to those used for the UV treatments, at 24:18°C, with a 14 h:10 h light-dark cycle.

A long-term feeding trial was used to assess the effects of UV-B-mediated changes in white clover composition on insect growth, development, and pupal weight. Groups of 12-14 neonate larvae were apportioned to 9 cm plastic petri dishes, one dish for each of seven pots per white clover population per UV treatment. Trifoliate leaves were clipped from potted plants and petioles inserted into 1.5 ml microcentrifuge tubes containing water. Leaves were placed into the petri dishes with larvae and replaced every 1-3 days. At one week of age, larvae showed signs of cannibalism, so seven surviving larvae were randomly selected from each petri dish and placed individually into dishes. Larval weights were recorded weekly until the onset of pupation. Upon pupation, pupal weight, sex, and development time (egg hatch to pupation) were recorded.

A short-term feeding trial was conducted with penultimate (fourth) instars to determine the effects of experimental treatments on larval feeding, growth rates, and food processing efficiencies. Penultimate instars were used because the insects were large enough to accurately measure consumption rates, and because the beginning and end of the penultimate stadium is demarcated by clearly defined moults. Insects were reared on white clover population 'Kopu' (grown in the low UV room) from egg hatch through the third stadium. Upon moulting into the fourth stadium, larvae were weighed and placed individually into 9 cm plastic petri dishes containing a weighed white clover trifoliate. Leaves were kept hydrated and replaced as described previously. Two insects were assayed for each of seven pots per white clover population and UV treatment. Upon moulting into the fifth stadium, larvae were frozen, then larvae, frass, and uneaten leaf tissue were freeze-dried and weighed. Calculation of average growth rate (AGR), average consumption rate (ACR), approximate digestibility (AD), and efficiency of conversion of ingested food (ECI) were done according to standard formulas (Waldbauer, 1968). Initial dry weights of larvae were estimated from a wet:dry conversion factor derived from a sample of 15 newly moulted fourth instars. Similar

conversion factors for leaves were obtained from leaf samples collected for chemical analyses.

Graphania mutans *bioassays*

G. mutans egg masses were obtained from gravid females light-trapped in the vicinity of Palmerston North, New Zealand. Bioassays were conducted following completion of the *S. litura* feeding assays, using the same procedures except as follows. For the long-term feeding trial, groups of 12-15 larvae were apportioned to 9 cm petri dishes. Larvae were transferred to 15 cm petri dishes as third instars. When larvae reached the fifth stadium the number was reduced to six per container and transferred to 1 L ventilated plastic boxes. For the short-term feeding assay, penultimate instar larvae were in the fifth stadium. One insect was assayed for each of nine pots per white clover population and UV treatment.

Statistical analysis

Effects of UV-B irradiation and white clover population were examined using the General Linear Model procedure in SAS, release 6.12 (SAS, 1996). For short-term feeding trials such as those employed, differences in insect performance variables may result from differences in initial larval weight. Analysis of variance revealed no significant differences in initial larval weight for insects in various treatments. Moreover, analysis of covariance (SAS, 1996) revealed that initial larval weight was not a significant covariate for the insect performance variables reported here as showing statistically significant effects.

Results

Foliar chemistry

White clover chemical composition varied in response to UV treatment and between populations. Concentrations of available carbohydrates declined an average of 22% in high-UV clover, but did not differ between populations (Fig. 7-1). Levels of neutral detergent fibre (NDF) declined 14% in high-UV 'Huia', but were unaffected in 'Tienshan' (significant UV x population interaction) (Fig. 7-1). Levels of nitrogen tended to increase in UV-B-treated plants and the amount of nitrogen was slightly, but significantly, higher in 'Huia' than in 'Tienshan' (Fig. 7-1). While UV-B did not change the proportion of acyanogenic to cyanogenic 'Huia' plants (15 plants each), cyanogenic activity showed a strong, 50% increase in response to UV-B treatment in cyanogenic 'Huia' (Table 7-1). The first cyanogenesis assay indicated that the 'Tienshan' population was acyanogenic. Application of linamarase to the acyanogenic plant tissues indicated that acyanogenesis could be due to deficiencies in the enzymatic steps of cyanide production, rather than to absence of substrates. The low levels of linamarase-catalysed cyanide production in 'Tienshan' increased under high UV, but this effect was only half as pronounced as that exhibited by acyanogenic 'Huia' plants supplied with linamarase (Table 7-1). The UV-B-induced cyanide levels in 'Tienshan' were the same as the constitutive, linamarase-catalysed cyanide levels in 'Huia'. Levels of UV-absorbing methanol-extractable compounds did not change in response to UV treatment (Table 7-1).

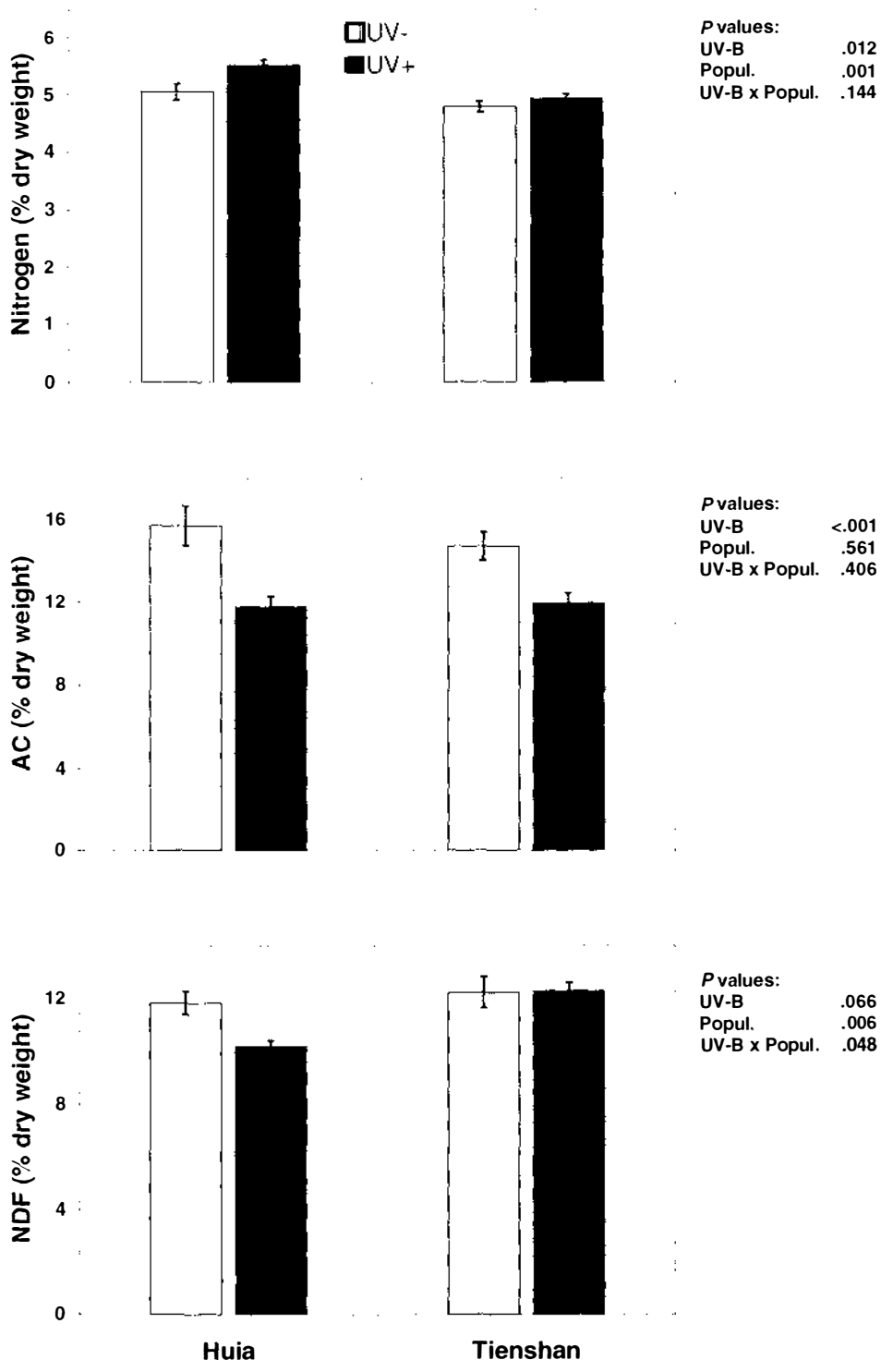


Fig. 7-1. Foliar chemical composition (mean + 1 SE) of two populations of white clover grown under low and high UV radiation. AC = available carbohydrates; NDF = neutral detergent fibre.

Table 7-1. Effects of UV treatment on cyanogenesis (HCN score mg^{-1} leaf dry matter) and UV-absorbing compounds (A_{300} mg^{-1} leaf dry matter) in white clover (mean ± 1 SE)

Treatment	Clover population	HCN score[a] mg^{-1}	Linamarase-catalysed HCN score[c] mg^{-1}	A_{300}[d] mg^{-1}
UV -	Huia	0.565 ± 0.053	0.350 ± 0.050	1.088 ± 0.037
UV -	Tienshan	nd[b]	0.256 ± 0.030	1.089 ± 0.042
UV +	Huia	0.850 ± 0.097	0.619 ± 0.047	1.090 ± 0.051
UV +	Tienshan	nd	0.358 ± 0.031	1.089 ± 0.042

[a] t-test revealed a significant effect of UV treatment on 'Huia' foliage, $P = 0.017$.
[b] nd = not detectable.
[c] ANOVA revealed effects of treatment and population (both $P < 0.001$) and of treatment × population ($P = 0.043$), on the linamarase-catalysed HCN score.
[d] ANOVA revealed no significant effects of UV or population on UV-absorbing compounds.

Spodoptera litura *performance*

The long-term bioassays showed that growth of larvae reared on high UV-treated foliage was reduced for insects fed the white clover population 'Huia' (Fig. 7-2). At two weeks of age, body weights of larvae in the high UV 'Huia' treatment were only 64% of those of larvae in the low UV 'Huia' treatment. In contrast, growth of larvae fed population 'Tienshan' over the same period was unaffected by UV treatment. No significant UV-B effect was observed on pupal weights, and only a slight prolongation of developmental time, especially in females (Fig. 7-3). White clover population only slightly affected larval development times, with insects fed 'Huia' requiring an additional day (relative to insects fed 'Tienshan') before the onset of pupation. Final pupal weights of males and females did not differ between white clover populations.

Short-term (fourth instar) bioassays revealed few if any effects of UV treatment on *S. litura* performance (Fig. 7-4 and Fig. 7-5). Approximate digestibility (AD) tended to improve in insects fed high-UV foliage, but the difference was slight and only marginally significant. Effects of white clover population were more pronounced. Insects fed 'Tienshan' had 20% higher consumption rates (ACR) but 10% lower food conversion efficiencies (ECI), compared with insects fed 'Huia'. Consequently, these insects exhibited a marginally significant, 10% increase in growth rates.

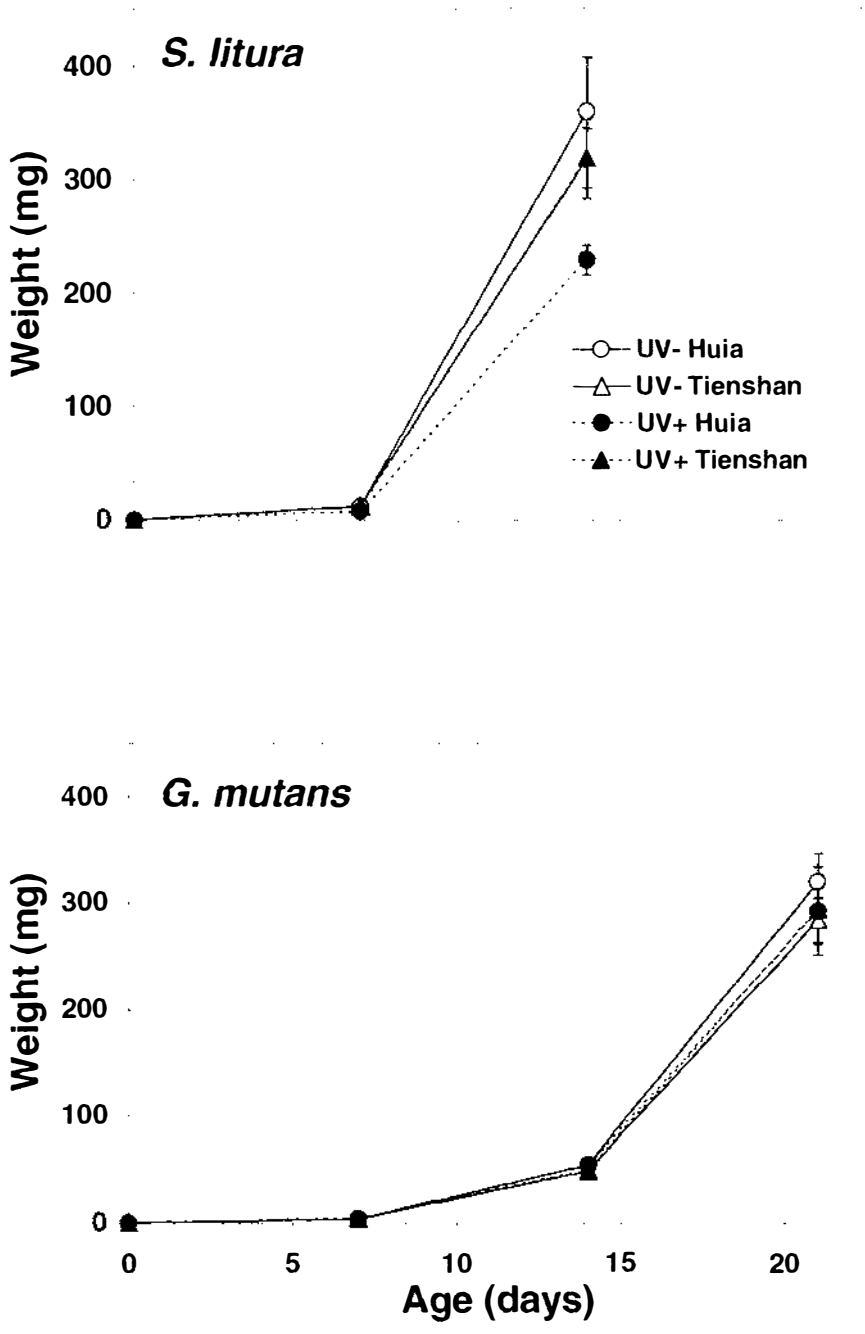


Fig. 7-2. Growth of *Spodoptera litura* and *Graphania mutans* larvae between egg hatch and pupation. Data collection at weekly intervals was terminated when the first larva in a treatment pupated. Vertical lines indicate ± 1 SE.

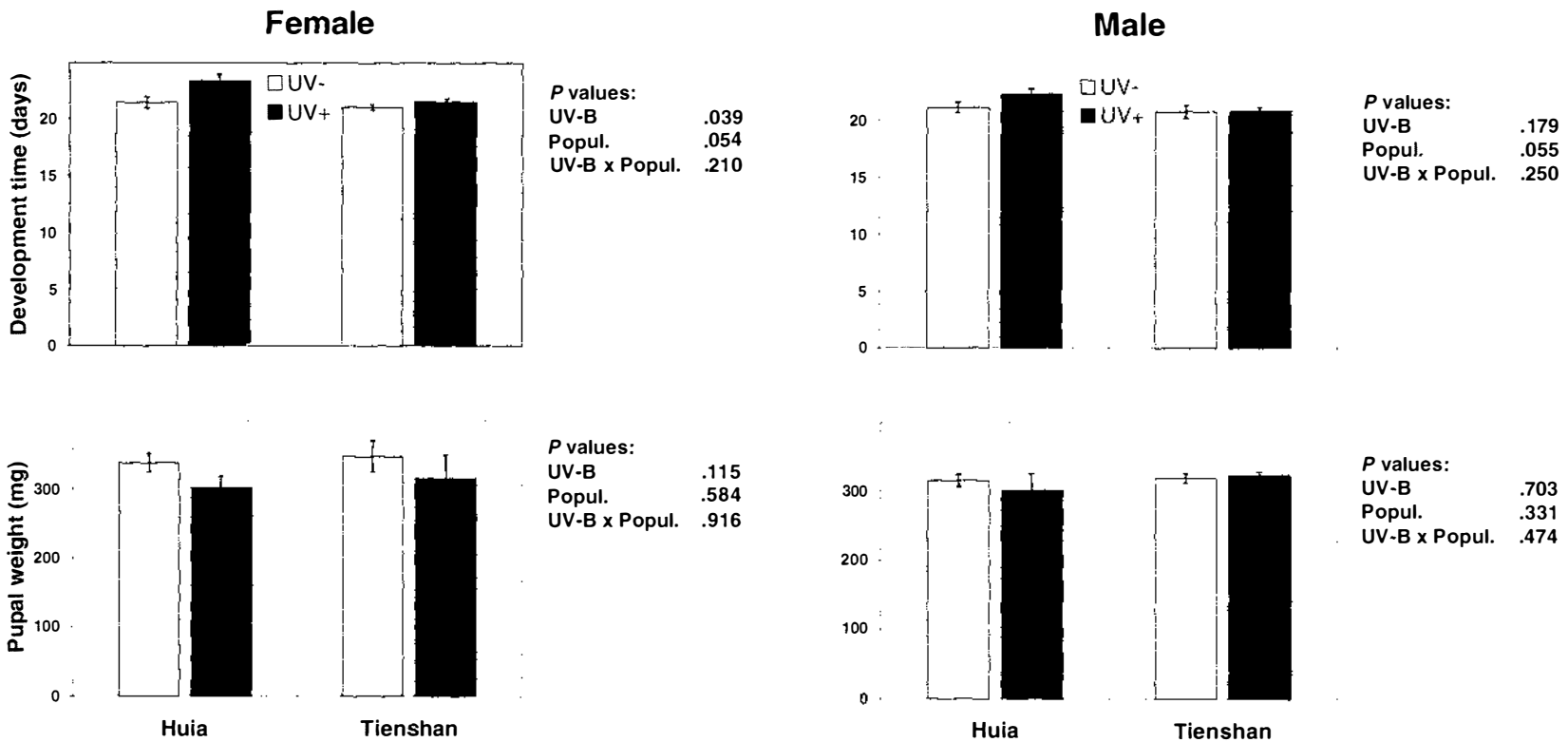


Fig. 7-3. Development times and pupal weights (mean + 1 SE) of *Spodoptera litura* reared on two populations of white clover grown under low and high UV radiation.

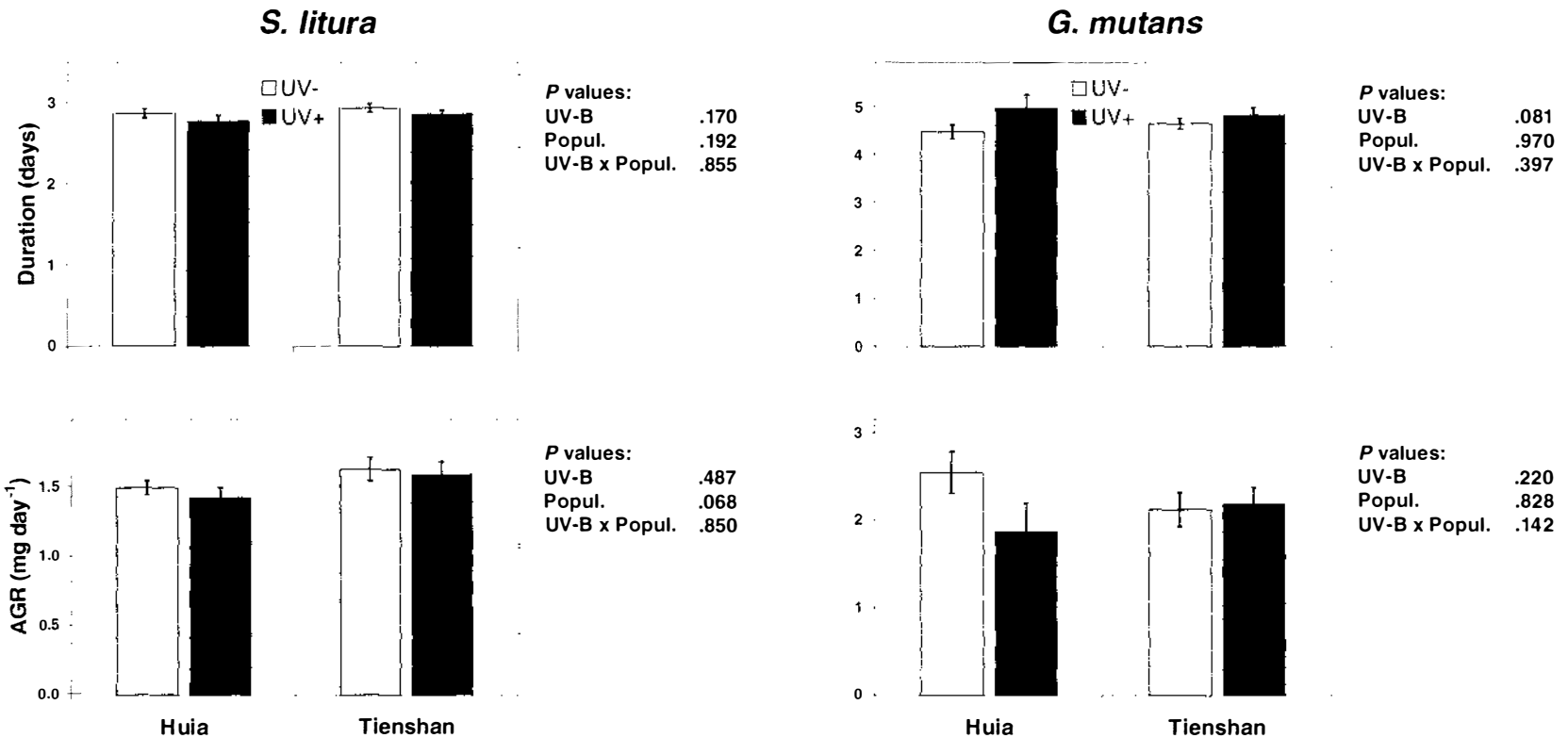


Fig. 7-4. Stadium duration and growth rates (mean + 1 SE) of penultimate instar *Spodoptera litura* and *Graphania mutans* reared on two populations of white clover grown under low and high UV radiation. AGR = average growth rate.

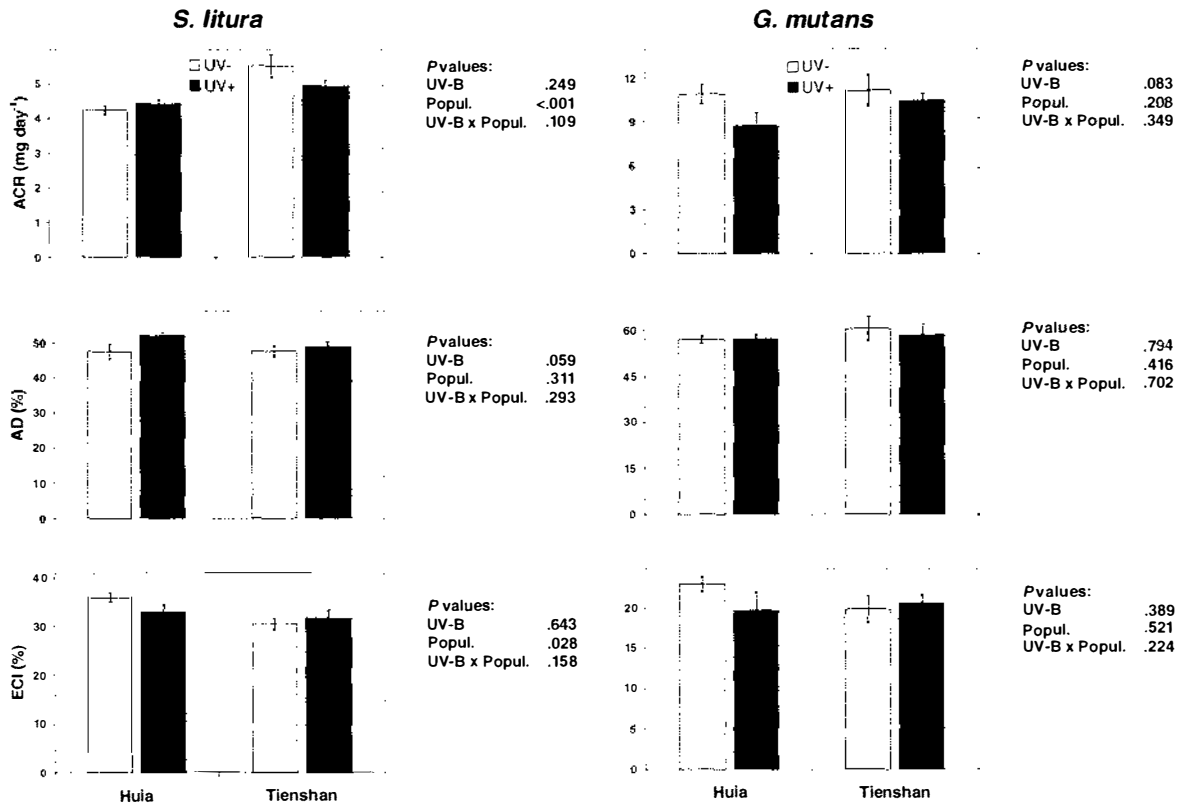


Fig. 7-5. Consumption rates and food utilisation efficiencies (mean + 1 SE) of penultimate instar *Spodoptera litura* and *Graphania mutans* reared on two populations of white clover grown under low and high UV radiation. ACR = average consumption rate; AD = approximate digestibility; ECI = efficiency of conversion of ingested food.

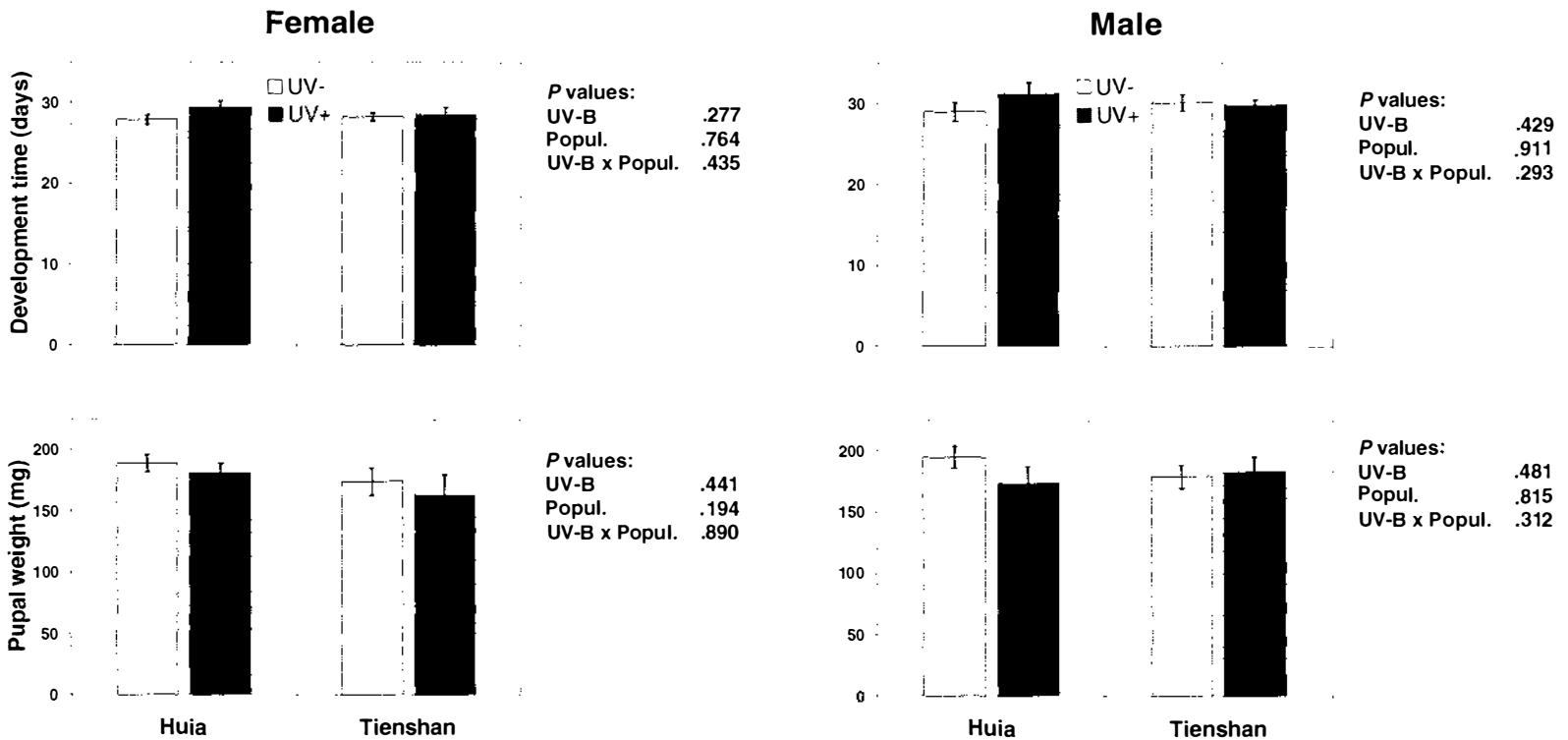


Fig. 7-6. Development times and pupal weights (mean + 1 SE) of *Graphania mutans* reared on two populations of white clover grown under low and high UV radiation.

Graphania mutans *performance*

Over the course of the three week period spanning egg hatch to onset of pupation, growth of *G. mutans* larvae was nearly identical on the four experimental diets (Fig. 7-2). Moreover, neither UV treatment nor white clover population influenced larval developmental periods or final pupal weights (Fig. 7-6). Relative to *S. litura, G. mutans* required an additional week for larval development and attained smaller pupal weights.

The short-term (fifth instar) feeding trials provided results similar to those of the long-term trials. Neither UV treatment nor white clover population affected insect growth rates or food utilisation efficiencies (Fig. 7-4 and Fig. 7-5). Trends toward longer development times and reduced consumption rates for insects on high-UV white clover were only marginally significant.

Discussion

Foliar chemistry related to primary metabolism

In this experiment, UV-B radiation elicited alterations in white clover composition that could be expected to affect the performance of herbivores. The largest effect was a decrease in available carbohydrate levels by more than 20% in both white clover populations (Fig. 7-1). This change was accompanied by decreased fibre levels in 'Huia'. To date, few studies have evaluated the effects of UV-B radiation on plant carbohydrate or fibre content. Decreased available carbohydrate concentrations at elevated UV-B levels have been reported for glucose in pea (Mackerness *et al.*, 1997), sucrose in mulberry (Yazawa *et al.*, 1992), and soluble carbohydrates in *Pinus* (Katzel *et al.*, 1996). In leaf litter of *Vaccinium uliginosum*, no effect of UV-B dose was observed on soluble carbohydrates, but a decrease in cellulose concentrations (Gehrke *et al.*, 1995). However, no effect of UV-B radiation was observed on hemicellulose content of *Gunnera magellanica* (Rousseaux *et al.*, 1998).

Overall levels of nitrogen in both white clover populations were quite high, well above the limiting threshold for most insects (Mattson, 1980; Slansky and Scriber, 1985). The slight increase in nitrogen concentrations in 'Huia' could therefore be regarded as less

important for herbivory than the aforementioned chemical changes, and the effect in 'Tienshan' was negligible (Fig. 7-1). Shifts of comparable magnitude have been reported for other legume species, such as soybean (Murali and Teramura, 1985a) and pea (Hatcher and Paul, 1994).

Foliar chemistry related to secondary metabolism

Together with the findings presented in Chapter 1 (Fig. 1-1), this is the first study to assess the impact of UV-B radiation on cyanogenesis. A clear UV-B-induced increase in cyanogenesis levels was found in cyanogenic plants (Table 7-1). The findings from linamarase supplementation indicate that UV-B-induced increases in cyanogenesis could primarily be due to increased substrate biosynthesis and are less dependent on increases in β-glucosidase concentration/activity. The possibility of UV-induced increases in white clover cyanogenic glycosides is notable given that these compounds are not derived from aromatic amino acids. The latter are products of the shikimic acid pathway, well known to be induced by UV radiation (Rozema *et al.*, 1997b). Linamarin and lotaustralin, however, are derived from the aliphatic amino acids valine and isoleucine (Seigler, 1998). Thus, their accumulation is mediated by the effects of UV-B on biochemical mechanisms other than those of the shikimic acid pathway. Interestingly, these findings suggest that in acyanogenic white clover genotypes, cyanoglucoside levels may rise under enhanced UV-B as well (Table 7-1).

The cyanogenesis levels in 'Huia' can be classified as moderate-high (Caradus and Woodfield, 1997; Crush and Caradus, 1995). The apparent acyanogenic nature of 'Tienshan' is consistent with its origin in a cold habitat, where frequent frost-related lesions of the tissue would disadvantage cyanogenic plants. The low linamarase-catalysed cyanide levels in 'Tienshan' could suggest lower constitutive levels of available cyanogenic substrates, which would be consistent with findings from other cyanogenesis studies on white clover populations originating in colder climates (Caradus and Eerens, 1992; Daday, 1965).

Foliar concentrations of flavonoids and related UV-absorbing compounds typically increase in response to UV-B radiation (Bornman *et al.*, 1997; Rozema *et al.*, 1997b). In contrast, no change was found in the UV-absorbing capacity of white clover grown

under elevated UV-B radiation (Table 7-1). This result is not without precedent, as several other recent studies (e.g. Rousseaux *et al.*, 1998; Salt *et al.*, 1998) have also failed to detect such a response. Moreover, the possibility cannot be ruled out that concentrations of some individual flavonoids or other phenolics changed, while overall UV-absorbing capacity remained unchanged. Another study in white clover revealed strongly increased flavonol levels under UV-B, concomitant with much less pronounced changes in UV-absorbing compounds (Chapter 5, Fig. 5-1 and Fig. 5-2).

In summary, results from the chemical analyses lend support to the pattern observed by others (e.g. Lavola *et al.*, 1998; Paul *et al.*, 1997), that phytochemical responses to UV-B radiation are compound-, species-, and population-specific. Such variability can be expected to influence the interactions of plants with higher trophic levels.

Insect Responses

Results from this research demonstrate that insect responses to UV-B-mediated changes in white clover chemical composition are specific to the particular plant-herbivore interaction investigated. The most pronounced effect observed was for growth of *S. litura* during the first two weeks of the larval developmental period (Fig. 7-2). Ultimately, however, UV-B-treated white clover had only a slight effect on development time of female *S. litura* and no effect on pupal weights of either insect species (Fig. 7-3 and Fig. 7-6). Because the fecundity of female Lepidoptera is generally correlated with pupal weight, the results suggest that consumption of UV-B-irradiated white clover is unlikely to affect reproductive success in *S. litura* or *G. mutans*. However, these experiments focussed on the first generation and thus possible cumulative effects over several generations cannot be ruled out.

The cause of decreased growth in young *S. litura* larvae reared on high-UV 'Huia' is unclear, but may be related to high cyanogenic activity. Cyanogenesis was elevated in high-UV 'Huia' (Table 7-1) and only for this treatment was larval performance reduced. Although cyanide is a broadly toxic constituent, some insects exhibit metabolic adaptations (e.g., β-cyanoalanine synthase activity) against it (Lindroth, 1991). In a related noctuid species, *S. eridania*, it was found that cyanide stimulated larval feeding

and growth (Brattsen *et al.*, 1983). Other findings suggest that cyanogenic glycosides in white clover can act as feeding deterrents to insect pests (Ellsbury *et al.*, 1992).

In contrast to insects feeding on cyanogenic 'Huia' plants, a cyanogenic effect for insects feeding on 'Tienshan' would only be possible in the presence of both appropriate amounts of cyanogenic plant substrates and of suitable β-glucosidases in the insect gut. While the presence of the latter has been reported for several lepidopteran species (Ferreira *et al.*, 1998), the low levels of linamarase-catalysed cyanide production in 'Tienshan' under high UV (Table 7-1) could suggest that the lack of insect response was due to insufficient amounts of available cyanogenic substrates. For the population 'Huia' it is proposed that *S. litura* required several weeks to habituate to higher levels of cyanogenic activity in high UV, after which growth accelerated and compensated for earlier growth suppression. *G. mutans* may not have exhibited reduced growth because it is better adapted than *S. litura* to cyanogenic compounds in its diet. *G. mutans* larvae are noted apple pests in New Zealand (Burnip *et al.*, 1995) and apple (*Malus pumilus*) produces cyanogenic glycosides such as amygdalin (Conn, 1979).

The lack of pronounced effects on insect performance in these studies could also be attributed to the overall excellent nutritional quality of the white clover diet, which exhibited relatively high nutrient concentrations and low fibre levels across all treatments, compared with levels for this species under field conditions (Rattray and Joyce, 1974; Wanjaiya *et al.*, 1993). It remains to be investigated whether significant UV-B-induced changes persist in the field, where compounds of relevance to nutrition are likely to occur at more limiting levels for herbivore growth. In addition, several white clover populations used in pasture ecosystems have higher constitutive levels of cyanogenesis compared to 'Huia' (Crush and Caradus, 1995) and a further UV-B-induced increase could be of relevance for insect feeding as well as mammalian herbivory. Consideration of ruminant sensitivity to cyanogenesis (e.g. Lehmann *et al.*, 1991) indicates a need for field experimentation under grazing conditions and concomitant inclusion of other stress forms (e.g., drought) which are likely to influence cyanogenesis and other aspects of white clover nutritive composition, and in turn nutrient utilisation by animals (Wanjaiya *et al.*, 1993).

Relatively few experiments have investigated the plant-mediated effects of UV radiation on insects, and results have varied among species and studies. With respect to consumption, no effect was found on feeding rates of *S. litura,* and only a slight trend toward reduced feeding in *G. mutans.* (Fig. 7-4 and Fig. 7-5) Other studies have documented the complete range of potential feeding responses to high-UV radiation, including decreases (Grant-Petersson and Renwick, 1996; Hatcher and Paul, 1994; Rousseaux *et al.*, 1998), no change (Grant-Petersson and Renwick, 1996), and increases (Lavola *et al.*, 1998). In terms of insect growth performance, little if any effect was found of high-UV radiation. Other researchers have reported reduced growth (Grant-Petersson and Renwick, 1996; McCloud and Berenbaum, 1994), no change (Grant-Petersson and Renwick, 1996; Stout *et al.*, 1998) and increased growth (Hatcher and Paul, 1994).

It is concluded that generalisations about the implications of higher UV-B radiation levels for plant biochemistry and herbivory must take account of genotypic differences in both plant and animal populations. The study shows that important differences may occur between populations of the same plant species in the biochemical response to UV-B radiation and in the subsequent effects on herbivore performance. Further research is required to understand the underlying functional basis for differential responses in both plants and animals given that UV-B effects on plant-insect interactions are species- and population-specific. These data suggest that a scheme for classifying the functional bases of plant-herbivore interactions should, amongst other attributes, take into account cyanogenic capacity, differential climatic adaptation and stress tolerance of plants.

Chapter 8. Responses to ultraviolet-B radiation in frequently defoliated *Trifolium repens* populations: differential growth, photochemistry and anatomy

White clover leaf structure: adaxial epidermal cell with underlying palisade mesophyll

This Chapter has been submitted to *Physiologia Plantarum*.

Abstract

This study examined responses to UV-B in two white clover populations exposed to frequent defoliation and 16 weeks supplementation with or without ultraviolet-B radiation (UV-B) of 13.3 kJ m^{-2} d^{-1} under controlled environmental conditions. Investigations included examinations of a number of leaf morphological characteristics, several chlorophyll fluorescence parameters as well as aspects of internal leaf structure and ultrastructure. In particular, it was aimed to explore whether possible differences in these features between the two white clover populations could be related to their UV-B sensitivity. Leaf growth decreased by 16% in the UV-B-sensitive cultivar 'Huia' under UV-B, while the tolerant ecotype 'Tienshan' was unaffected. UV-B effects on leaf dry mass and leaf size were similar, leading to no change of specific leaf mass (SLM). Percentage leaf dry mass (PDM) was reduced by 8% across populations in response to UV-B. Chlorophyll fluorescence measurements showed no photochemical sensitivity to UV-B. Results from light- and electron microscopy suggest some regulatory, rather than destructive UV-B effects on leaf ultrastructure. While the integrity of epidermal and mesophyll cells and of chloroplasts was maintained, it appeared that there was a decrease in overall starch content. There were also structural differences between the two white clover populations, suggesting higher, more densely packed dome-shaped epidermal cells for 'Tienshan'. It is concluded that differential UV-B-sensitivity in white clover populations can also occur under frequent defoliation. UV-B tolerance could be related to structural epidermal characteristics inherent to the slower-growing stress-tolerant population.

Introduction

Increased ultraviolet-B radiation (UV-B, 290-315 nm) resulting from depletion of the stratospheric ozone layer can influence plant processes either through direct damage or via various regulatory effects (Rozema *et al.*, 1999). The latter includes UV-B-generated morphological changes such as smaller leaf size (Matthew *et al.*, 1996) decreased leaf weight (Krizek *et al.*, 1998) as well as differential UV-B effects on these two attributes, yielding higher specific leaf mass (leaf dry mass based on leaf size, SLM) (Filella and Penuelas, 1999). There are also indications that leaf dry mass based on leaf fresh mass (percentage leaf dry matter, PDM) could be affected by UV-B (Wand, 1995).

Direct UV-B-induced plant damage has often been observed in alterations of plant photochemistry, particularly photosystem II (PSII) function, measured by chlorophyll fluorescence. This includes UV-B-generated decreases in the intrinsic efficiency of PS II (F_v/F_m) (Nikolopoulos *et al.*, 1995), reduced photochemical yield ($\Delta F/F'_m$) (Reuber *et al.*, 1996a) and decreased photochemical quenching (q_P) (Reuber *et al.*, 1996b). There are also reports of altered non-photochemical quenching (NPQ) in response to UV-B (Reuber *et al.*, 1996a).

Regulatory functions of UV-B for plant structure include increased number of cell layers (Nagel *et al.*, 1998), epidermal thickening (Hunt and McNeil, 1998) as well as palisade cell elongation (Cen and Bornman, 1990; Nagel *et al.*, 1998). Other studies found a general UV-B-induced decrease in epidermal cell surface size and that small constitutive cell size (i.e. measured under no UV-B supplementation) was linked to UV-B tolerance (Chapter 2, Fig. 2-2b). UV-B-induced damage symptoms on a structural level include collapsed epidermal cells (Cen and Bornman, 1990; Santos *et al.*, 1993) and intracellular disintegration (Santos *et al.*, 1993). Disruption of chloroplast ultrastructure has also been described as a main target of UV-B irradiation in several studies (e.g. Caasi-Lit *et al.*, 1997; He *et al.*, 1994).

The pasture legume white clover is frequently exposed to defoliation pressure from grazing animals. Little is known on the effectiveness of UV-B on plants exposed to continuous defoliation. Frequent defoliation stress can affect various plant functions, for instance light interception, stolon and root development or carbohydrate accumulation (Hart, 1987). A field study in a New Zealand sward including white clover showed UV-B-induced reductions in pasture growth under sheep grazing. In other controlled environment studies (Chapter 3, Fig. 3-2a) differential UV-B effects were observed in white populations subjected to infrequent defoliation. These findings provided a framework suggesting a fundamental relationship between UV-B responsiveness one the one hand and the productivity of white clover populations and their habitat on the other.

The present study aims at further expanding this framework by examining the two white clover populations 'Huia' and 'Tienshan' after frequent defoliation during 16 weeks growth under UV-B. 'Huia' is a commonly used white clover cultivar bred for agricultural productivity, while 'Tienshan' is a slow-growing ecotype adapted to multiple stresses. Morphological, photochemical and structural attributes and their responses to UV-B were examined. In particular, this study intended to explore whether possible between-population differences in these features could be linked to differential UV-B sensitivity.

Materials and Methods

Experimental design, plant cultivation and UV irradiation treatments were identical to those described in Chapter 7. The plants were subjected to the UV treatments for 29 days prior to initiation of defoliation pressure. From then on, all plants experienced frequent periods of defoliation during the collection of leaf material used for bioassays as described in Chapter 7. Plants were allowed to recover from defoliation stress for 10 days before leaves were harvested for morphological, photochemical and structural examinations.

Leaf material

Leaf size was measured at the end of the experiment with a LI-COR Model 3100 area meter on four fully open distal leaves per plant. Laminae were subsequently dried at 80°C for 48 h for dry mass determination. The ratio of dry mass over leaf area was calculated to give specific leaf mass (SLM), while leaf dry mass percentage (PDM) was calculated from (dry mass/fresh mass)*100. These measurements were conducted on ten plants (replicates) for each white clover population under each UV treatment.

Chlorophyll fluorescence

Chlorophyll fluorescence measurements were performed on fully open distal leaflets of 5 replicate samples for each population under each treatment condition using the same methods as described in Chapter 5. Effects of UV-B irradiation and white clover

population on leaf attributes and chlorophyll fluorescence parameters were examined with the General Analysis of Variance procedure in GENSTAT (Genstat, 1993).

Structural studies

In order to explore possible structural differences in response to UV-B, duplicate leaf samples per population under each treatment condition were prepared as follows. Strips (1 mm wide) were cut perpendicular to the main leaf vein from the central part of the middle leaflet of fully open distal white clover leaves. Sections were fixed in 3% glutaraldehyde + 2% formaldehyde in 0.1M phosphate buffer (pH 7.2) (Karnovsky, 1965). After vacuum infiltration, the sections were kept in the fixative for 24 hours at 4°C. This was followed by three washes in fresh buffer for 30 min. The samples were subsequently post-fixed for 1 hour at room temperature in 1% osmium tetroxide in the same buffer. Following three further buffer washes the specimens were dehydrated in an acetone series (25, 50, 75, 95, 100%), infiltrated and embedded in Procure 812 epoxy resin (cured at 60°C for 48 hours).

For light microscopy, sections of 1.0 μm thickness were cut with a diamond knife in an ultra-microtome (Reichert-Jung Ultracut E). Samples were stained with toluidine blue and mounted in immersion oil on glass slides. Light microscopy was done using a Zeiss Axiophot photomicroscope (Zeiss, Oberkochen, Germany) with 35 mm colour film. For transmission electron microscopy, sections of 0.1 μm thickness were cut using the ultra-microtome. Grid-mounted samples were stained for 4 min with saturated uranyl acetate in 50% ethanol, followed by washing in 50% ethanol and subsequently in distilled water. Staining with lead citrate (4 min) (Venable and Coggleshall, 1965) was followed by a final washing step in distilled water. Transmission electron microscopy was conducted with a Philips 201C electron microscope operating at 60kV. Figures presented here are typical representations of findings from light- and electron microscopy.

Results

After 16 week exposure, the most notable UV-B-induced plant morphological change was a decrease in leaf dry matter (Table 8-1). Least significant difference analysis (LSD, at $P < 0.05$) showed that this decrease was significant in the cultivar 'Huia' (-16%), while 'Tienshan' was unaffected by UV-B (Table 8-1). In the other leaf growth attributes measured here the white clover populations did not display significant sensitivity to UV-B, even though 'Tienshan' consistently appeared less affected by UV-B than 'Huia' (Table 8-1). However, there were significant inherent leaf morphological differences between the two white clover populations, with 'Huia' displaying larger leaves than 'Tienshan' (Table 8-1). Percent leaf dry mass (PDM) decreased by about 8% across populations under UV-B (Table 8-1). No differences between UV-B treatments or white clover populations were found for specific leaf mass (SLM).

Chlorophyll fluorescence parameters generally showed no population-specific or UV-B-induced differences (Table 8-2). Apart from a small rise in intrinsic efficiency of photosystem II (F_v/F_m) by about 2%, changes in photochemical yield ($\Delta F/F'_m$), and photochemical (q_P) as well as non-photochemical quenching (NPQ) were not significant (Table 8-2). Adaxial epidermal cells were intact, with lens-shaped (domed) cells that were particularly convex in 'Tienshan' (Fig. 8-1). Mesophyll cells were also undamaged and there were 1-2 layers of palisade tissue and about 4 layers of spongy mesophyll in all samples (Fig. 8-1). Chloroplast integrity was maintained with intact external (chloroplast envelope) and internal membranes, showing a regular pattern of the grana and stroma thylakoid network (Fig. 8-2). Starch accumulation could be observed in all samples (Fig. 8-2), but examination of the leaf cross-section suggested a decrease in starch content under UV-B (Fig. 8-1).

Table 8-1. Morphological attributes (mean ± 1 SE) in white clover grown with (UV+) and without (UV-) supplementation of 13.3 kJ m^{-2} d^{-1} UV-B. Statistical information includes significances for main and interaction effects as well as the least significant differences (LSD) for the UV-B × population term. DM = dry mass; PDM = percent leaf dry mass; SLM = specific leaf mass.

Treatment	Clover population	Leaf DM (mg)	Leaf size (cm^2)	Petiole length (mm)	SLM (mg cm^{-2})	PDM (%)
UV -	Huia	19.1 ± 1.24	7.01 ± 0.503	98.6 ± 7.19	2.75 ± 0.063	19.7 ± 0.26
UV -	Tienshan	11.8 ± 0.56	4.36 ± 0.320	65.9 ± 4.95	2.74 ± 0.080	20.4 ± 0.60
UV +	Huia	16.0 ± 0.78	6.20 ± 0.251	92.0 ± 4.13	2.57 ± 0.059	17.7 ± 0.39
UV +	Tienshan	11.4 ± 0.79	4.41 ± 0.450	70.3 ± 5.28	2.67 ± 0.109	19.1 ± 0.59
UV		0.050	0.349	0.839	0.130	0.002
Population		< 0.001	< 0.001	< 0.001	0.522	0.038
UV × population		0.116	0.282	0.322	0.511	0.418
LSD ($P < 0.05$)		2.48	1.129	15.78	0.230	1.38

Table 8-2. Photochemical parameters (mean ± 1 SE) in white clover grown with (UV+) and without (UV-) supplementation of 13.3 kJ m^{-2} d^{-1} UV-B. Statistical information includes significances for main and interaction effects as well as the least significant differences (LSD) for the UV-B × population term. $\Delta F/F'_m$ = photochemical yield; F_v/F_m = intrinsic efficiency of photosystem II; NPQ = non-photochemical quenching; q_P = photochemical quenching.

Treatment	Clover population	F_v/F_m	$\Delta F/F'_m$	q_P	NPQ
UV -	Huia	0.814 ± 0.0027	0.620 ± 0.0071	0.879 ± 0.0089	0.591 ± 0.0332
UV -	Tienshan	0.803 ± 0.0069	0.625 ± 0.0096	0.897 ± 0.0067	0.547 ± 0.0913
UV +	Huia	0.825 ± 0.0028	0.626 ± 0.0169	0.871 ± 0.0204	0.609 ± 0.0340
UV +	Tienshan	0.827 ± 0.0016	0.638 ± 0.0070	0.878 ± 0.0159	0.582 ± 0.0475
UV		< 0.001	0.402	0.342	0.645
Population		0.274	0.416	0.377	0.541
UV × population		0.111	0.761	0.731	0.881
LSD ($P < 0.05$)		0.0121	0.0328	0.0422	0.1700

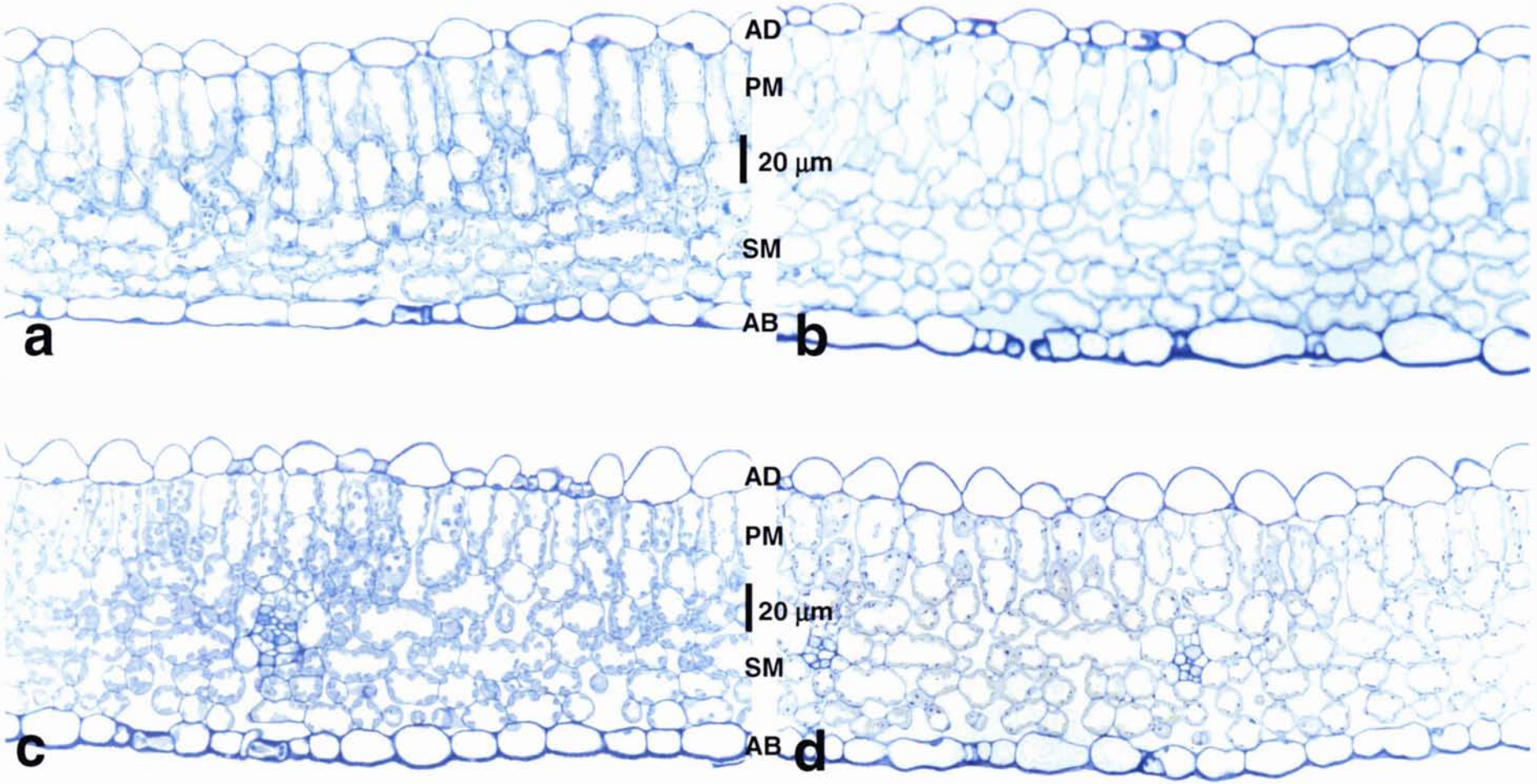


Fig. 8-1. Light micrographs of transverse white clover leaflet sections stained with toluidine blue. Starch grains appear as small granules inside the mesophyll chloroplasts. (a) 'Huia' UV-; (b) 'Huia' UV+; (c) 'Tienshan' UV-; (d) 'Tienshan' UV+. AB = abaxial epidermal cells; AD = adaxial epidermal cells; PM = palisade mesophyll; SM = spongy mesophyll.

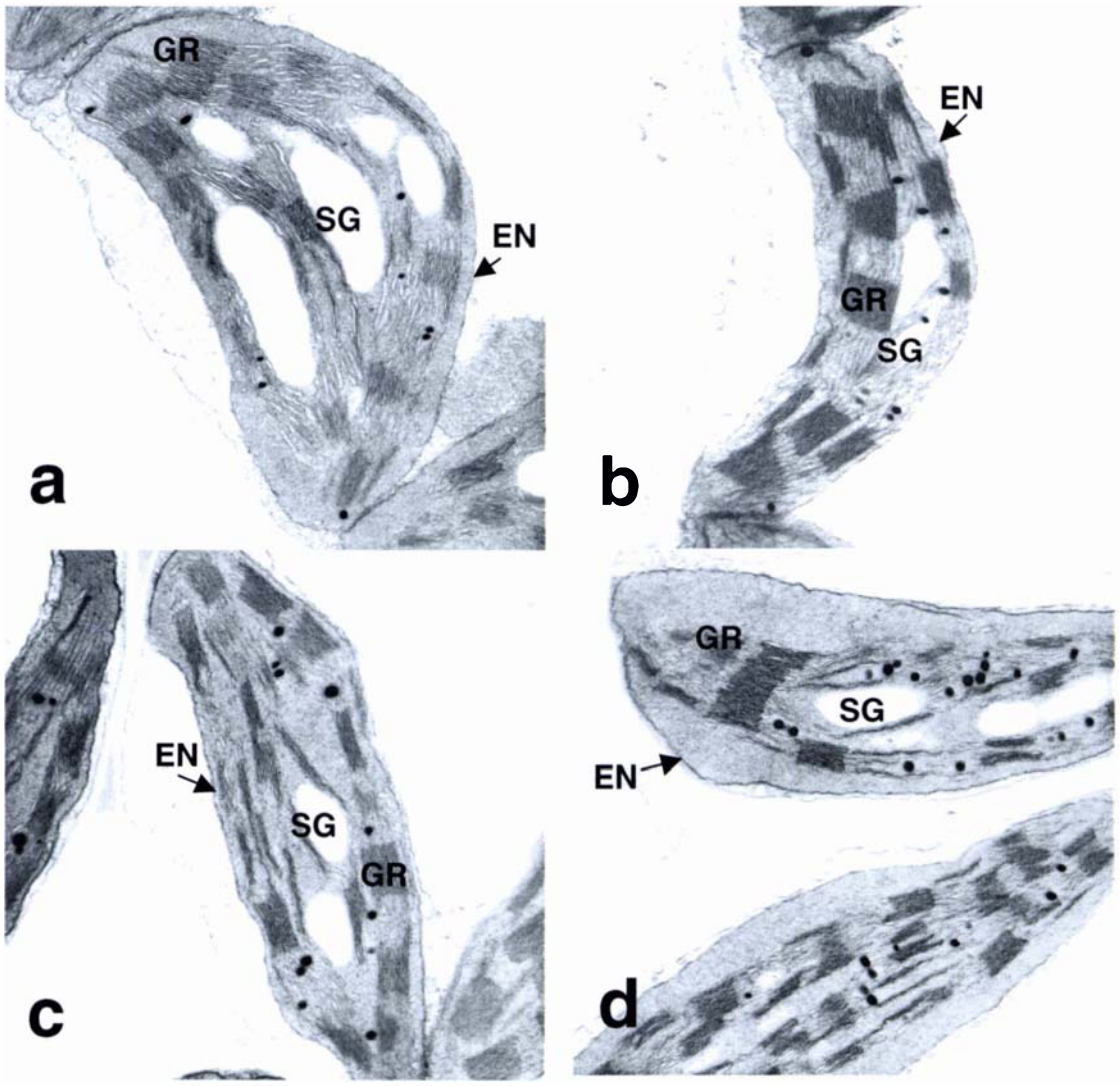


Fig. 8-2. Transmission electron micrographs of transverse sections of white clover leaflets exposed to supplementation with (UV+) or without (UV-) 13.3 kJ m^{-2} d^{-1} UV-B. Intact chloroplasts. (a) 'Huia' UV-; (b) 'Huia' UV+; (c) 'Tienshan' UV-; (d) 'Tienshan' UV+. EN = chloroplast envelope; GR = granum; SG = starch grain. × 16 000.

Discussion

Leaf growth and photochemistry

Constitutive and UV-B-generated differences in leaf growth were observed between the two white clover populations (Table 8-1). In particular, leaf mass in the larger-leafed cultivar 'Huia' was susceptible to UV-B, while the less productive ecotype 'Tienshan' showed no such response after 16 weeks UV-B exposure (Table 8-1). Population-specific differences in leaf growth responses under UV-B have also been observed in other studies (Dai *et al.*, 1994; Furness *et al.*, 1999). Contrary to 'Huia', 'Tienshan' also shows tolerance to a number of other stress forms, including low temperature, low fertility and drought (Chapter 3, Table 3-1). The direct link between higher constitutive leaf mass in 'Huia' and UV-B-induced decreases in leaf dry matter production suggests that previously established links of plant and habitat productivity to UV-B sensitivity (Chapter 3) can also be observed under strong defoliation pressure.

While no UV-B-induced change was observed in specific leaf mass (SLM), leaf dry mass percentage (PDM) was decreased under UV-B (Table 8-1). Similarly, examinations of 38 South African plant species show no change in SLM, but decreases in PDM under increased UV-B levels (Wand, 1995). A number of studies reported increases in SLM under UV-B (Britz and Adamse, 1994; Li *et al.*, 1998), but decreases have also been observed (Balakumar *et al.*, 1993; Krizek *et al.*, 1997a). A lack of consistent changes in SLM has led to suggestions that this attribute may be a less reliable indicator of UV-B stress (Fiscus *et al.*, 1996). Other findings point at higher relative importance for leaf dry matter content, rather than SLM for the prediction of stress tolerance (Wilson *et al.*, 1999). The observed decreases in PDM under UV-B could be indicative of improved water status. Other studies in white clover found UV-B-induced increases in leaf water potential (Chapter 5, Fig. 5-3). Improved water relations under UV-B could be due to a number of factors, including improved root:shoot ratios, stomatal limitations or biosynthesis of stress proteins and osmolytes (Schmidt *et al.*, 2000; Teramura *et al.*, 1984a).

Measurements of various chlorophyll fluorescence parameters showed that photosystem II (PSII) photochemistry did not differ between the white clover populations and was

not negatively affected by UV-B (Table 8-2). Inhibition of growth without negative effects on PSII photochemistry has also been observed in other studies (Gonzalez *et al.*, 1996; Nogues *et al.*, 1998). The present findings suggest adequate protection of PSII function, which could be mediated by photoprotective mechanisms such as the presence of UV-B-protective pigmentation (Allen *et al.*, 1998).

Structural studies

Results from light microscopy (Fig. 8-1) and transmission electron microscopy (Fig. 8-2) suggest no marked structural damage in the UV-B-treated white clover samples. Epidermal cells were intact and displayed the distinctive lens shape on the adaxial surface described for this cell type in white clover (Vogelmann *et al.*, 1996) (Fig. 8-1). In particular, 'Tienshan' displayed a more convex curvature at the adaxial surface than 'Huia' (Fig. 8-1). This could be a function of the previously established differences in cell sizes between the two populations, with smaller size (lower surface area) and thus more dense packing of cells in 'Tienshan' (Chapter 2, Fig. 2-2b). Accordingly, the microscopic investigations suggest smaller periclinal cell diameter for 'Tienshan' epidermal cells, and in addition these cells also appear to be higher than in 'Huia' (Fig. 8-1). Previously, an inverse correlation was found between UV-B sensitivity and constitutive cell size (Chapter 2, Fig. 2-2b) and the present findings may indicate that this could also be due to differential cell height (and thus epidermal thickness). A longer vertical pathlength in 'Tienshan' for incoming UV-B radiation could constitute a UV-B-protective feature (Gorton and Vogelmann, 1996).

The pronounced lens shape particularly in 'Tienshan' could further affect leaf optical properties of relevance for UV-B transmission. Differences in epidermal cell size and shape have been related to differences in light focussing and scattering (Gorton and Vogelmann, 1996; Vogelmann *et al.*, 1996). In particular, epidermal cells with a small radius of curvature generate a focal plane that lies in the epidermis or in the upper mesophyll (Vogelmann *et al.*, 1996). A survey of 47 plant species showed that white clover had one of the smallest radius of curvature (highly convex outer cell surface), focussing light just below the epidermal layer (Vogelmann *et al.*, 1996). Higher convexity of 'Tienshan' epidermal cells could suggest increased focussing of light and of the accompanying UV-B radiation. This would further increase optical pathlength and

concentrate UV-B towards the epidermal cells which usually contain specific UV-B-attenuating pigmentation such as flavonoids (Gorton and Vogelmann, 1996). Another study revealed higher UV-B-induced levels of specific flavonoids in 'Tienshan', compared to 'Huia' (Chapter 6, Fig. 6-2).

Epidermal focussing has been noted in early publications (Haberlandt, 1914), but to date little is known about the adaptive and physiological significance of epidermal lens effects, on a species or population level and between habitats (Vogelmann *et al.*, 1996). The studies in white clover suggest that epidermal differences of relevance for light penetration can occur between plant populations and that this could have adaptive ecological significance for UV-B responsiveness. In particular, epidermal cell geometry in the less productive, stress-tolerant white clover ecotype 'Tienshan' displayed properties which could contribute towards the protection from UV-B. Further studies are required to measure whether UV-B penetration through the white clover epidermis, particularly directly underneath central cell regions, differs between white clover populations. This, in conjunction with measurements of UV-attenuating pigmentation in the epidermal layer would assist in the verification of differential epidermal cell shape contributing to UV-B protection.

Palisade and spongy mesophyll cells also were intact and showed a similar number of layers in all samples (Fig. 8-1). This is in contrast to other findings, reporting disruption of intracellular structure and of cell walls (Caasi-Lit *et al.*, 1997). The 1-2 layers of palisade tissue and approximately 4 layers of spongy mesophyll cells are in accordance with previous observations for white clover (Thomas, 1987a). Integrity of the photosynthetic tissue as well as of chloroplasts (Fig. 8-2) conforms with the findings from chlorophyll fluorescence studies, showing no damaging UV-B effects on PSII function (Table 8-2). Other reports link chloroplast membrane damage to inhibition of PSII function (Brandle *et al.*, 1977; He *et al.*, 1994). Integrity of granal stacks is of particular importance in this regard, as they contain most of the PSII complexes. A study in rice reported UV-B-generated ultrastructural disruption of granal stacks and of the chloroplast envelope (Caasi-Lit *et al.*, 1997). In the present study there were no indications of differential vesicle formation or thylakoid dilation between UV-B treatments, as observed elsewhere (Brandle *et al.*, 1977; He *et al.*, 1994). Structural chloroplast damage in *Pisum* (thylakoid dilation, disruption in the envelope) (He *et al.*,

1994) was accompanied by the accumulation of large starch grains, also observed in maize (Santos *et al.*, 1993). Contrasting to this, examinations of the white clover samples suggest a decrease in starch content under UV-B (Fig. 8-1). UV-B-induced decreases in starch volumes were also recorded in *Brassica* (Fagerberg and Bornman, 1997). Such decreases were compared to a shade-type response and it was suggested that this could be a result of starch mobilisation as well as of decreased starch synthesis (Fagerberg and Bornman, 1997). The UV-B-induced decreases in starch content observed here are reflected by decreased levels of available leaf carbohydrates (starch and soluble sugars, see Fig. 7-1 in Chapter 7). Decreases in soluble sugars could point towards reduced substrate availability for starch synthesis under UV-B. Reduced starch accumulation in the absence of chloroplast damage can be considered a regulatory, rather than destructive consequence of UV-B irradiation.

In conclusion, exploration of leaf internal leaf structures suggests structural integrity and inherent differences in epidermal cell structure between the two white clover populations. This warrants further structural examinations on a larger scale, also investigating quantitatively the observations made here on a qualitative basis. The present study adds further information to a framework linking plant specialisation to UV-B responsiveness. In particular, this suggests a relationship between population-specific epidermal cell geometry and UV-B sensitivity. Between-population variation in leaf mass and UV-B sensitivity under frequent defoliation could suggest that UV-B effects may also occur under grazing pressure.

Chapter 9. Discussion

This Chapter will be submitted in part to *Trends in Ecology and Evolution.*

These studies address a number of issues that provide new information to the field of ultraviolet-B plant research. Thus far there had been a lack of comprehensive investigations on pasture species, particularly on a population level. Inclusion of a diverse range of white clover populations added weight and generality to the findings and facilitated comparability among functional plant types, independent from confounding species-specific differences. This also allowed testing whether stress-tolerant plants would be UV-B resistant, a hypothesis previously not examined in detail. Investigations ranged from specific biochemical responses to trophic interactions under controlled environmental conditions. A number of morphological and physiological UV-B effects were detected. There were significant population differences in several attributes, both constitutively and in response to UV-B. Differences in UV-B sensitivity of the white clover populations were linked to constitutive features of plant productivity, morphology and tolerance to other forms of stress as well as to UV-B-induced physiological attributes. UV-B responsiveness was mitigated by drought and by duration of UV-B exposure. Population-specific UV-B responsiveness could also be detected in frequently defoliated white clover leaves and differentially affected the performance of insect herbivores. In the following section, a framework is proposed, based on constitutive and UV-B-induced attributes of the white clover populations in relation to their UV-B responsiveness.

A functional framework to synthesise results

The results from this series of investigations can be drawn together in a conceptual model, suggesting a fundamental relationship of UV-B responsiveness to the productivity of white clover populations and of their habitat (Fig. 9-1). This is further linked to a number of morphological-physiological attributes (Fig. 9-1). The key steps contributing to this overall result are summarised in Table 9-1, using the plant attributes listed in Fig. 9-2 and the individual experimental approaches in Table 9-2 to Table 9-4.

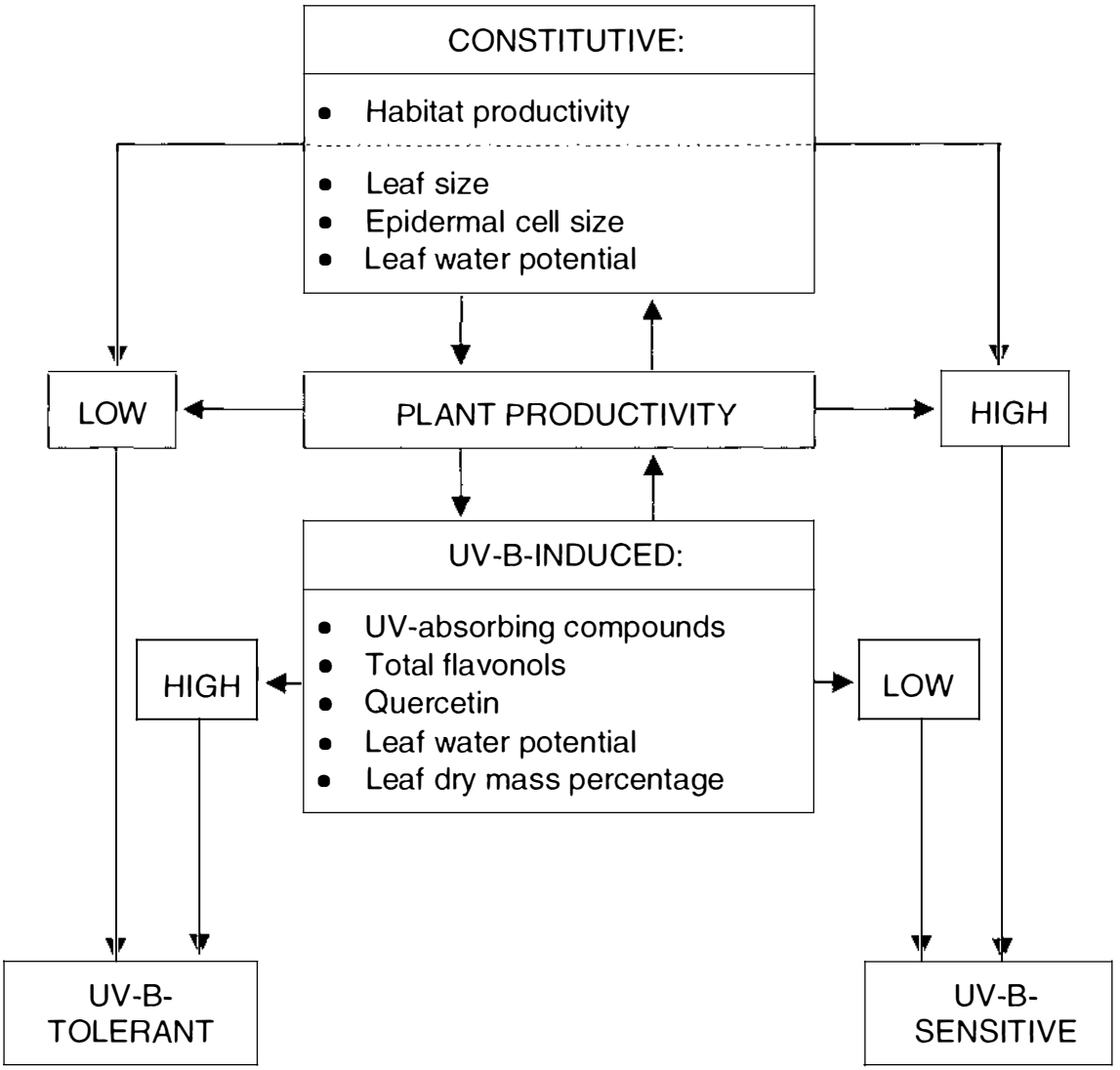


Fig. 9-1. Model linking plant productivity and related morphological and physiological features in white clover to UV-B responsiveness.

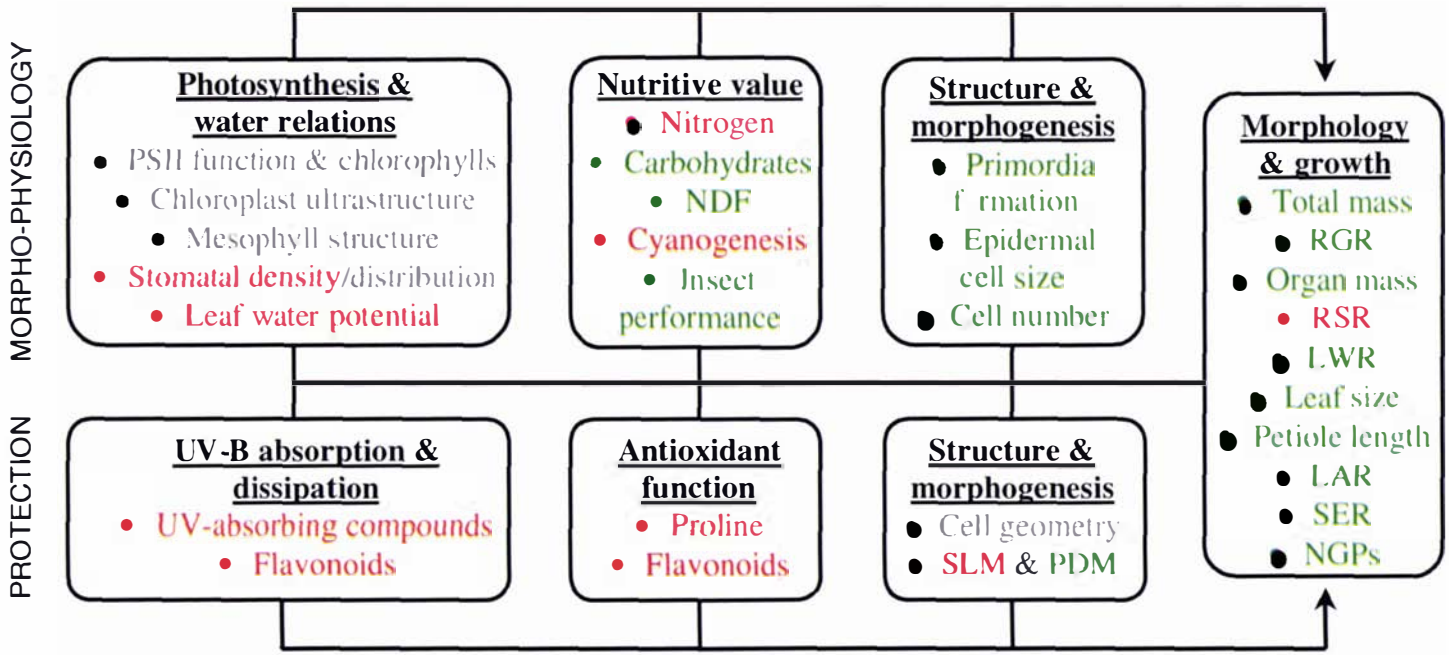


Fig. 9-2. Scheme of the current research project, based on morphological and physiological attributes in white clover. Changes in plant attributes due to UV-B are marked. Red font: increases; green font: decreases; grey font: no change. LAR = leaf appearance rate; LWR = leaf weight ratio; NDF = neutral detergent fibre; NGPs = number of growing points; PDM = percent leaf dry mass; PSII = photosystem II; RGR = relative growth rate; RSR = root:shoot ratio; SER = stolon elongation rate; SLM = specific leaf mass.

Table 9-1. Overall features of the series of investigations.

Plants	• Comprehensive investigation of UV-B responsiveness in white clover, a pasture species exposed to markedly higher UV-B levels in the New Zealand pasture ecosystem than at comparable latitudes in the northern hemisphere. • Inclusion of a diverse range of white clover populations, including developed cultivars as well as ecotypes from the wild, testing whether habitat adaptation relates to UV-B responsiveness. • Detailed background information on 26 white clover populations, including ecological and breeding characteristics.
⬇	
Attributes (Fig. 9-2)	• Examination of a wide variety of growth and morphological features, including growth of total plants (total growth and growth rates) and of main organs, including leaves (mass, size, number, petiole length), stolons (mass, length, number) and roots (mass), as well as growth within (PDM, SLM) and between (RSR, LWR) plant organs. • Microscopy studies (stereo microscopy, LM, TEM) on various structural and ultrastructural aspects of buds, epidermal cells, stomata and mesophyll cells. • Physiological measurements of primary (levels of nitrogen, carbohydrates, starch, fibre, proline, chlorophylls, PSII function) and secondary metabolism (accumulation of flavonoids, UV-absorbing compounds, cyanogenesis), complemented by measurements of ψ_L.
⬇	
Factors	• Investigation of UV-B effects on these attributes in combination with other abiotic and biotic factors, including population comparisons, drought, time, defoliation and herbivory.
⬇	
Methods	• Application of statistical methodology examining the various variables and factors, including principal components analysis to provide integrated representation of numerous UV-B responses and residual maximum likelihood analysis to study interactions of UV-B with time, white clover populations and drought.
⬇	
Links	• Establishment of constitutive and UV-B-induced differences among the various white clover populations in a number of plant attributes. • Relationship of many of these differences to constitutive biomass accumulation and in turn to UV-B sensitivity.
⬇	
Key outcome	• Classification of UV-B responsiveness according to functional population types, separated by attributes linked to plant productivity

Table 9-2. Key aspects from large-scale screening of 26 white clover populations for their UV-B susceptibility based on 17 plant attributes.

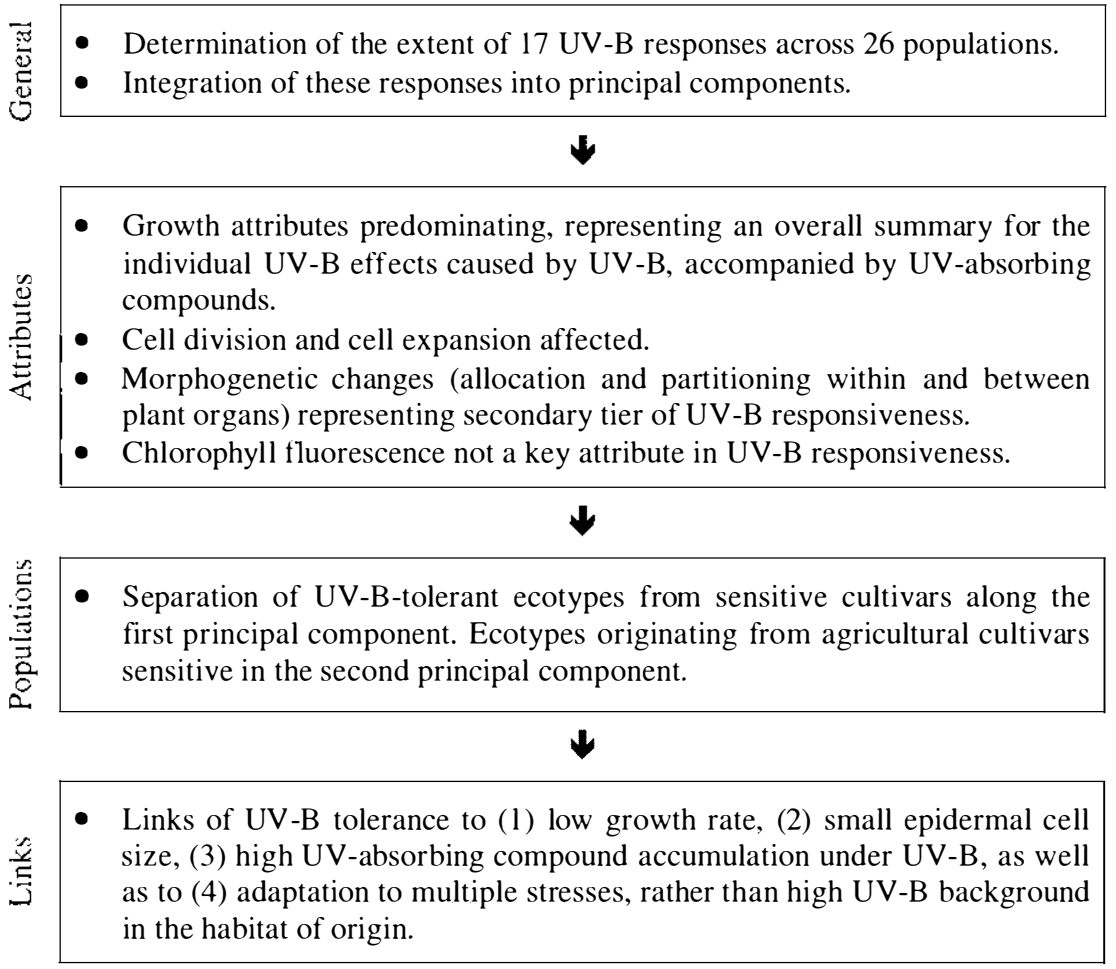


Table 9-3. Key aspects from examinations of UV-B susceptibility in interaction with drought and of flavonoid responses in nine white clover populations.

General	• Examination of the UV-B × drought interaction on a population level. • Examination of a number of attributes only occasionally studied or novel in UV-B research on a population level, including accumulation of specific flavonoid compounds and of proline, ψ_L, PSII fluorescence quenching, PDM, leaf primordia formation in the bud, stolon growth.
⬇	
Morphology	• Confirmation of the overall picture obtained from large-scale screening. • Reduction of all growth attributes by UV-B, including productivity, leaf growth, rates of stolon elongation and leaf appearance, leaf primordia formation. • Both leaf size and leaf number affected by UV-B. • UV-B effects on aboveground structures shielded from UV-B, suggesting indirect impacts on cell division. • Dry matter allocation within the leaf (PDM, SLM) generally not affected.
⬇	
Physiology	• Identification of the flavonoid structures involved in the white clover UV-B response. • Flavonols, particularly quercetin glycosides involved. • Total flavonol responses and quercetin accumulation well-conserved on a population level: increased in all populations. • General increase in the quercetin:kaempferol ratio. • Flavonols much more increased than UV-absorbing compounds. • No UV-B effect on fluorescence quenching, suggesting well-protected PSII function. • Consistent UV-B-induced increases for ψ_L in drought-stressed plants and for higher proline levels in well-watered conditions.
⬇	
Populations	• No overall association between UV-B habitat and flavonol production, but between habitat productivity and accumulation of specific flavonoid compounds: highest flavonol and quercetin levels in stress-tolerant ecotypes, suggesting particular ecophysiological roles for dihydroxylated flavonoids in stress protection. • Similarly, UV-B-induced increases in UV-absorbing compounds for stress-tolerant Asian ecotypes. • Increases of ψ_L in these ecotypes, indicating UV-B-elicited advantages in aspects of water retention.

Populations (contd.)

- Increased proline levels and decreased ψ_L under UV-B in white clover populations with intrinsically low proline and high ψ_L levels.
- High intrinsic ψ_L in populations with low PDM under well-watered conditions, also related to lower proline levels.
- Highest proline levels (under drought) and lowest intrinsic ψ_L (in well-watered conditions) for stress-tolerant populations of low productivity.
- Inverse constitutive relationship between quercetin glycoside accumulation and ψ_L.

Drought

- Much stronger influence of drought for primary productivity: decreases in total growth and leaf growth, in chlorophyll levels and ψ_L, increases in leaf dry matter allocation (SLM and PDM), NPQ and proline. Little effect on UV-absorbing compounds and flavonols.

Combination of UV-B and drought

- Amelioration of UV-B responses by drought: only marginal or non-significant UV-B effects on morphology and growth, concomitant with no population differences in the UV-B response.
- Particularly beneficial effect of drought for UV-B sensitivity in productive white clover cultivars.
- Primary role of UV-B for flavonol induction, but pronounced synergistic interaction with drought.
- Synergistic interaction also for UV-absorbing compounds and ψ_L.
- UV-B-induced improvements of water relations under drought: PDM decreased, while ψ_L was consistently increased across populations.
- Possible amelioration of UV-B sensitivity mediated by (1) drought alone via (a) delays in a number of growth attributes, (b) increases in SLM, PDM, proline, as well as by (2) synergistic UV-B × drought interaction via increases in UV-absorbing compounds, flavonols (especially quercetin glycosides) and ψ_L.

Time

- Decreased UV-B sensitivity with increasing duration of drought (additive effect in the early drought phase, antagonistic after four weeks) and with increasing exposure to UV-B.
- Similar UV-B sensitivity ranking among the white clover populations over time.

Links

- Links to UV-B growth tolerance via (1) constitutive features, including low productivity, small leaf size, low ψ_L, and (2) via UV-B-induced features, including increases in UV-absorbing compounds, in flavonols, and in ψ_L, as well as higher quercetin (but not kaempferol) glycoside accumulation and higher PDM (rather than SLM) under UV-B.

Table 9-4. Key aspects from bioassay studies and examinations of UV-B susceptibility of frequently defoliated white clover populations.

General	• Investigation of species differences in pasture insect pests feeding on UV-B-treated leaves of two different white clover populations.
Attributes	• UV-B effects on primary metabolic and morphological processes: increased nitrogen levels; decreases in fibre, PDM, carbohydrate, starch levels, reduced leaf growth; PSII function unaffected. • Regulatory, rather than destructive UV-B effects on leaf ultrastructure. • Secondary metabolism: cyanogenesis strongly increased by UV-B in cyanogenic cultivar 'Huia'. • Increased substrate biosynthesis for cyanogenesis in both cyanogenic and acyanogenic genotypes; no UV-B effect on UV-absorbing compounds.
Links	• Differential insect responses to UV-B-mediated foliar changes in 'Huia', temporary sensitivity in *Spodoptera litura* may be related to increased cyanide production. • Other UV-B-induced changes in the performance of both insect species generally small. • UV-B tolerance in white clover may be related to structural epidermal characteristics inherent to the slower-growing stress-tolerant white clover population.

Characteristic features of the model in Fig. 9-1 first emerged from the application of multivariate statistical techniques, integrating 17 UV-B responses across 26 white clover populations after $2^1/_2$ weeks of exposure to UV-B (Table 9-2). Further investigations tested these findings under longer-term UV-B irradiation (12 weeks) and in combination with drought stress (Table 9-3). Thus far, very little functional information had been available on ecological interactions between plant stress forms in conjunction with populations of the same species (Krupa *et al.*, 1998a). Very few studies had previously examined the combination of UV-B with drought on a population level. The observations from this work (Table 9-3) suggest major physiological UV-B action occurs via indirect effects on processes linked to secondary metabolism, rather than processes linked to primary photosynthetic function. This supports current views attributing UV-B an indirect, regulatory role in physiological and ecological processes, mediated by polyphenolics (Rozema *et al.*, 1999).

Compared to productive cultivars, slow-growing, UV-B-tolerant populations from stress-exposed habitats displayed greater ability for such biochemical mechanisms of UV-B protection. In addition to deficiencies in biochemical defense mechanisms, stress susceptibility in the cultivars was also related to plant features contributing towards resource acquisition, e.g. larger leaves (Table 9-3). While less pronounced, this could also be observed under frequent defoliation (Table 9-4). Sensitivity of one generalist lepidopteran species, but not of another to UV-B-treated 'Huia' foliage points at the possibility of highly specific plant-herbivore interactions (Table 9-4).

These findings contributed to a conceptual framework for UV-B responsiveness, suggesting (1) a general advantage of lower habitat and plant productivity for increased capacity in biochemical UV-B protection, while (2) specialisation towards biomass gain leads to morphological change resulting in pronounced reductions of plant productivity (Fig. 9-1). Comparisons of morphological and physiological mechanisms showed that UV-B responsiveness is mainly linked to constitutive differences (i.e. measured under no UV-B supplementation) in morphology and growth among the populations, while it is more the UV-B-induced physiological differences that confer UV-B tolerance (Fig. 9-1). A metabolic cost of a plant strategy towards constitutive and UV-B-induced protection against UV-B may be lower investment of carbon allocation towards primary constitutive productivity. The results from these studies thus propose functional

population types for UV-B responsiveness in white clover, separating stress-tolerant, slow-growing ecotypes with greater ability for biochemical mechanisms of UV-B protection from agricultural white clover cultivars showing the opposite characteristics.

This model is consistent with ecological models of stress tolerance, proposing distinct plant types depending on primary plant strategies (Diaz and Cabido, 1997; Diaz *et al.*, 1999; Grime, 1979; Grime *et al.*, 1997; Poorter and Garnier, 1999). Plant stress responses can be classified into two main categories: morphological and physiological (Bradshaw, 1965). The latter category is seen of particular relevance for stress-tolerant plant types (Grime *et al.*, 1997). Stress tolerators are less likely to use morphological changes as a strategy against stress, because of generally slower growth and biomass turnover. Instead, cellular acclimation through biochemical adjustments would be the expected primary strategy (Grime and Campbell, 1991). On the other hand, plant types specialised towards higher resource capture would be expected to display a larger degree of morphological plasticity in response to stress, mainly due to changes in the meristematic regions of the plant (Grime and Campbell, 1991). A common route for expression of the latter would be decreased growth (Grime and Campbell, 1991).

Analysis of 24 vegetative and regenerative traits in 100 Argentinean plant species over a wide range of climatic conditions demonstrated trade-offs between investment in growth on the one hand and allocation to stress protection on the other (Diaz and Cabido, 1997). Attribute-environment linkages allow particular traits or trait combinations to be used for predictions of responses within and between plant species under changing climatic conditions (Diaz *et al.*, 1999). Compared to their less productive counterparts, fast growing plant types usually have higher leaf area and less relative root mass, contributing to larger carbon gains per unit plant mass (Lambers and Poorter, 1992). They often have higher photosynthetic rates, higher levels of organic nitrogen and minerals as well as lower carbon levels (Lambers and Poorter, 1992; Poorter and Garnier, 1999). Slow-growing plant types on the other hand often contain higher levels of cell wall compounds, starch and secondary metabolites, display increased leaf longevity, higher leaf density as well as higher dry matter content, relative to fresh mass (Lambers and Poorter, 1992; Poorter and Garnier, 1999). These features are seen to confer general tolerance to abiotic and biotic stress factors (Lambers and Poorter, 1992; Poorter and Garnier, 1999).

Many of these characteristics were found in the slower-growing white clover populations originating in stress-exposed habitats. The relationships between morpho-physiological plant attributes, productivity and UV-B responsiveness observed here propose linkages previously recognised in other fields of stress research. The relationship to habitat productivity confirms for UV-B responsiveness suggestions that resistances to a number of stress forms can be predicted from each other (MacGillivray and Grime, 1995). Furthermore, it appears that all three classes of UV-B protection (Beggs *et al.*, 1986) may be present in the white clover UV-B response, including repair (e.g. antioxidant functions by flavonols), attenuation (e.g. UV-absorbing compounds) and growth delay (e.g. reduced cell division).

Comparisons between experiments

Seven white clover populations from the initial screening programme (Chapter 2) were also included in the drought interaction experiments (Chapters 3-5) and in investigations of flavonoid responses (Chapter 6). These included the ecotypes 'Sarikamis', 'Tienshan' and 'Häggås' as well as the cultivars 'Huia' , 'Kopu', 'Prestige' and 'Haifa'. Chapters 7 and 8 further investigated 'Tienshan' and 'Huia' attributes during the bioassay experiments. With each experiment, the plants experienced progressively increased duration of UV-B exposure, from $2^1/_2$ weeks to 16 weeks (Table 9-5). The 12-week trials (Chapters 3-6) also included three-week preconditioning with gradual increases in UV-B. Nevertheless, the general pattern of UV-B sensitivity was consistent, irrespective of duration in UV-B application, developmental stage of the original plant material, soil type or defoliation treatment (Table 9-5). UV-B-generated dry matter reduction for the populations in the screening experiment ranked similarly to that in well-watered conditions during the stress interaction trials (Fig. 9-3). This was even more pronounced when comparisons were made across water treatments ($r = 0.759$, $P < 0.05$). There was also no apparent overall change in effect when ultraviolet-A radiation (UV-A) was included in the control treatment of the bioassay studies (Table 9-5). However, the previously established time-dependency of UV-B responsiveness (Fig. 3-4) seemed further confirmed by the observation that after the longest period of UV-B exposure (16 weeks, Chapter 8), only some aspects of leaf growth were affected by UV-B (Table 8-1).

Table 9-5. General comparison between UV-B application, plant conditions and UV-B sensitivity in three main experimental approaches studying UV-B effects on white clover. Daily UV-B levels in all experiments were 13.3 kJ m^{-2} d^{-1}.

Feature	Large-scale screening	Stress interaction studies and flavonoid responses	Bioassay studies
Duration of UV-B treatment	$2^1/_2$ weeks	12 weeks (with preconditioning)	16 weeks
UV-A included	No	No	Yes
Plant material	Stolon cuttings	Seedlings	Stolon cuttings
Soil material	Sand	Sand	Soil
Defoliation	No	Infrequent	Frequent
UV-B sensitivity	yes	yes	yes
Differences in UV-B sensitivity among populations	yes	yes	yes
UV-B tolerance for ecotypes	yes	yes	yes

Constitutive plant growth attributes correlated well between experiments, exemplified by the productivity measurements in Fig. 9-4. Similarly close relationships could be found for leaf dry mass (r = 0.977, P < 0.001) and leaf size (r = 0.975, P < 0.001). Population-specific differences in leaf growth were also maintained after 16 weeks growth under controlled environmental conditions (Chapter 8, Table 8-1). Plant attributes not affected after long-term UV-B irradiation (SLM and F_v/F_m,) were only marginally or not significantly related between experiments. This further indicates little importance of these attributes in white clover UV-B responsiveness. However, UV-B-induced accumulation of UV-absorbing compounds (r = 0.721, P = 0.068) and relative increases in these levels were significantly related between experiments (Fig. 9-5). It was of interest to note that UV-absorbing compounds did not show UV-B-induced increases in 'Tienshan' in Chapter 7 (Table 7-1). It is possible that the frequent defoliation stress led to alterations in leaf composition away from UV-absorbing compounds.

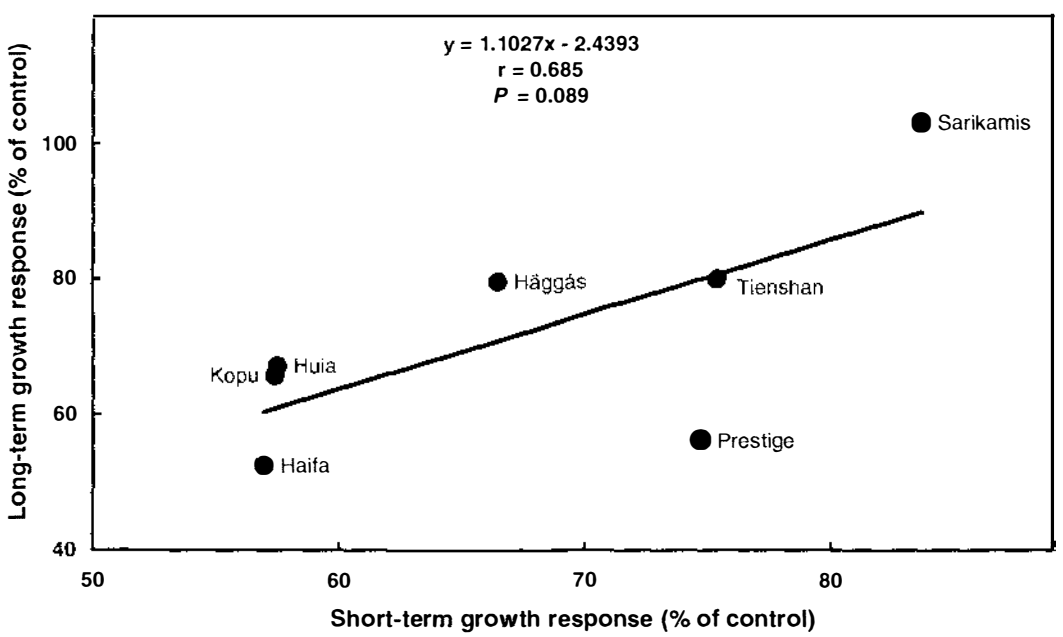


Fig. 9-3. Comparison of growth responses under well-watered conditions of seven white clover populations exposed to supplementation with 13.3 kJ m^{-2} d^{-1} UV-B for 12 weeks (ordinate) versus a $2^1/_2$ week screening experiment (abscissa). The growth response is expressed as relative growth (dry matter production under UV-B expressed as a percentage of that of the control).

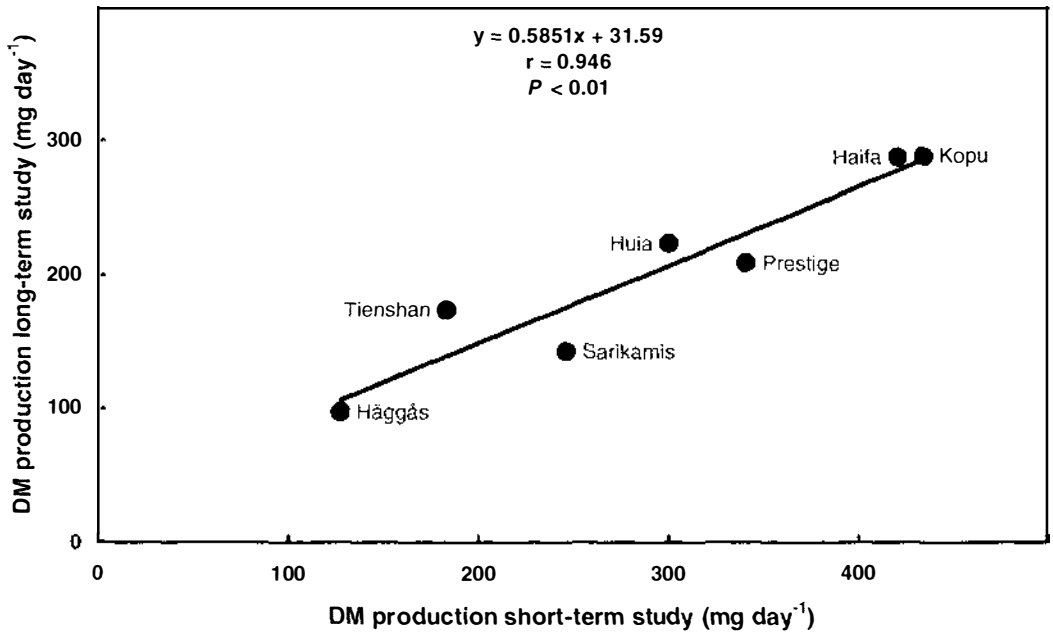


Fig. 9-4. Comparison of constitutive growth (dry mass production per day under well-watered conditions without UV-B supplementation) of seven white clover populations grown under controlled environmental conditions for 12 weeks (ordinate) versus a $2^{1}/_{2}$ week screening experiment (abscissa). DM = dry mass.

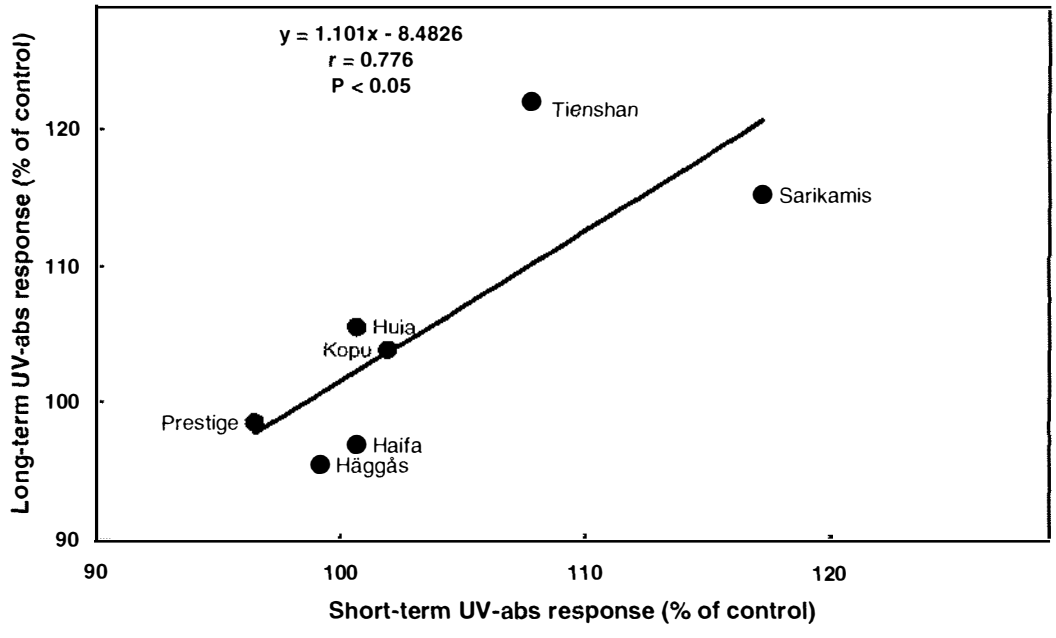


Fig. 9-5. Comparison of UV-absorbing compound (UV-abs, measured at 300 nm) responses under well-watered conditions of seven white clover populations exposed to supplementation with 13.3 kJ m^{-2} d^{-1} UV-B for 12 weeks (ordinate) versus a $2^{1}/_{2}$ week screening experiment (abscissa).

Context and limitations of the findings

The daily indoor photosynthetic photon flux (PPF) levels used in these experiments were about half of the average daily mid-summer PPF (Greer and Laing, 1992) and the plant-weighted UV-B dose was 58% higher, compared to the daily clear-sky mid-summer UV-B dose above Palmerston North (McKenzie, 1991). These experiments used a 'square-wave' approach, subjecting plants to constant UV-B exposure, rather than to the gradual increase in the field. The control was a 'zero' UV-B treatment which in most cases (Chapters 2-6) had no UV-A supplementation. As a consequence, these conditions are likely to lead to larger UV-B effects on plants, compared to outdoor conditions. For example, higher PPF and UV-A levels contribute to protective functions such as photorepair and flavonoid production (Nogues *et al.*, 1998).

Nevertheless, such artificial indoor conditions are often used to study mechanisms involved in UV-B responses (Allen *et al.*, 1997; Delong and Steffen, 1997; Lavola *et al.*, 1998; Nogues *et al.*, 1998; Olsson *et al.*, 2000; Olsson *et al.*, 1999). This approach was chosen (1) to clearly expose genetic differences among white clover populations in response to UV-B, (2) to discern possible functional mechanisms underlying differential UV-B responsiveness, and (3) to examine specific stress interactions without confounding effects by other environmental variables. Screening experiments examining UV-B responses in a number of populations are seen to be particularly practicable under controlled conditions (Corlett *et al.*, 1997). This is due to a number of factors, e.g. control of population-specific environmental interactions but also in consideration of the time and expense otherwise involved for field experimentation. Furthermore, similar UV-B responsiveness was observed among the white clover populations after preconditioning with UV-B by gradually increasing UV-B levels over three weeks (Chapter 3). Differential UV-B sensitivity within white clover was also maintained when plant attributes under UV-B were compared to a UV-A control (Table 9-5). The zero UV-B control can be used to discern UV-B effects per se, which would not be possible with two levels of UV-B irradiation but no zero control.

There are merits as well as limitations in the approach used here and caution will be necessary to extrapolate the findings from these studies to the field. The usefulness of laboratory data in predicting plant stress responses has been highlighted in a number of

studies on plant functional types (MacGillivray and Grime, 1995; Poorter and Garnier, 1999). One major function of the present experiments was to generate hypotheses leading to the development of a model for UV-B responsiveness, which can in consequence be tested in the field. Such investigations are presently being conducted.

A further limitation in these studies is that much of the evidence concerning linkages to UV-B responsiveness is correlative. However, there are also other indications for similar relationships from comparative studies between the cultivar 'Huia' and the mutant breeding line 'Red' (Fig. 9-6). The latter population was selected from 'Huia' phenotypes with high anthocyanin content. The comparison within a very similar genotypic range strengthens the evidence that there may be metabolic trade-offs between biochemical defense mechanisms and plant productivity. The results showed 25% higher constitutive levels of UV-absorbing compounds in 'Red' ($P < 0.01$) and a similar difference in growth rates, with higher productivity for 'Huia' ($P < 0.001$) Fig. 9-6a,c. Differences of cell size could not be assessed as these were similar between the two populations (Fig. 9-6d), which may be related to their common ancestry. However, constitutive leaf sizes were markedly smaller in 'Red' ($P < 0.001$) (Fig. 9-6b). Given the similarity in cell sizes, this therefore is a consequence of reduced cell numbers in 'Red', suggesting that potential morpho-physiological trade-offs between the two populations act at the level of cell division, rather than cell expansion.

'Red' displayed higher UV-absorbing compound levels than 'Huia', irrespective of UV-B treatment (Fig. 9-6c). Thus 'Red' did not increase levels of UV-absorbing compounds in response to UV-B, which could be due to the high constitutive values, already providing sufficient UV-B protection. Comparisons of UV-B effects on the productivity of the two populations clearly showed differential UV-B susceptibility with tolerance for 'Red' ($P < 0.01$) (Fig. 9-6a). The general picture emerging from the comparisons between the 'Red' mutant and its mother population 'Huia' is a reflection of the findings from the other studies in this project, suggesting UV-B tolerance for the slow-growing, small-leafed population with high UV-absorbing compound production. This is further supported by examinations of a green (anthocyanin-poor) and a red (anthocyanin-rich) *Coleus* population (Burger and Edwards, 1996). UV-B tolerance for the red population was related to protection by anthocyanins, e.g. UV-B absorption due to acylation with cinnamic acids (Burger and Edwards, 1996).

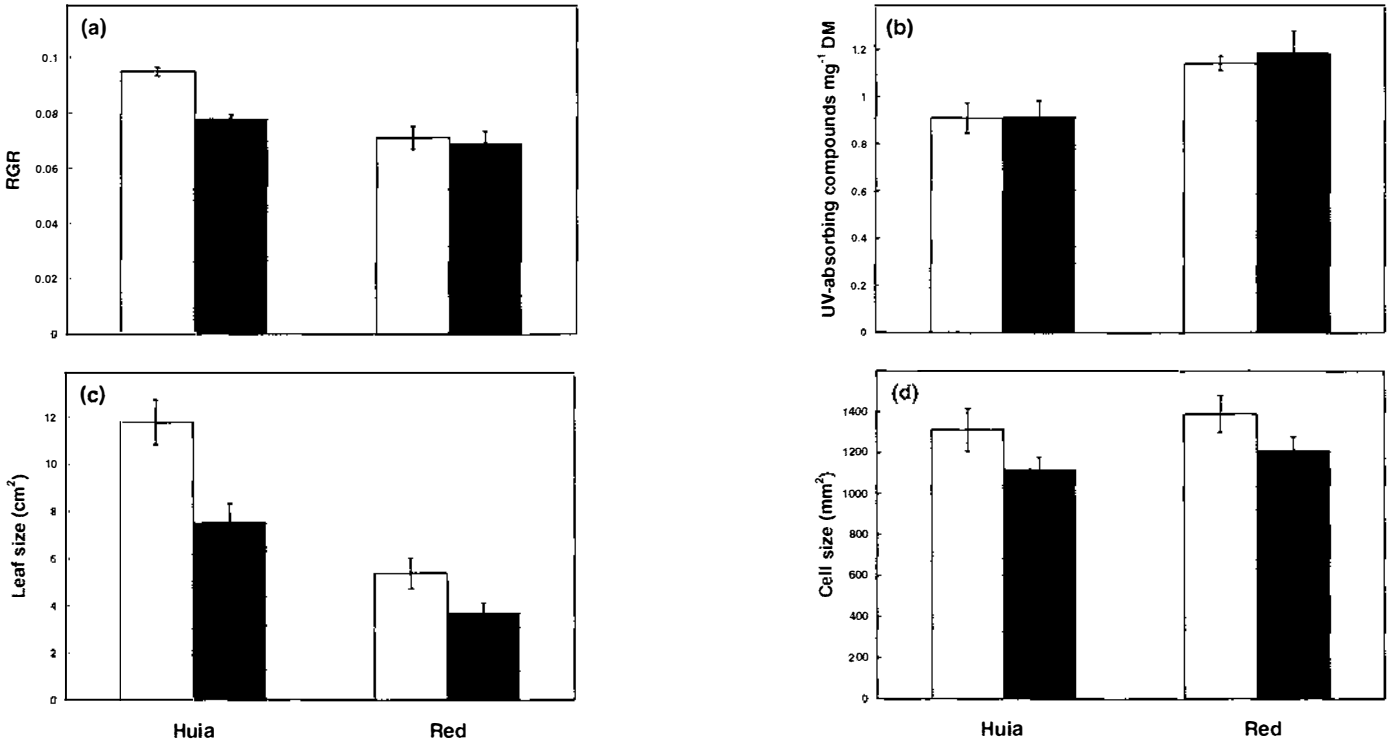


Fig. 9-6. Morpho-physiological attributes in the white clover populations 'Huia' and 'Red', grown with (closed columns, ■) or without (open columns, □) supplementation of 13.3 kJ m^{-2} d^{-1} UV-B; (a) relative growth rate (log-transformed increment of total plant biomass per day), (b) leaf size, (c) UV-absorbing compounds (d) cell size. DM = dry matter.

Perspectives and future studies

This work portends a range of future studies, from sub-cellular to ecosystem levels. UV-B-induced growth reductions occurred in the absence of damaging effects on chlorophyll accumulation or of marked inhibition of light conversion in the photosystem II reaction centre. While it is possible that other photosynthetic processes such as CO_2 fixation could still be affected by UV-B, growth reductions in the absence of any primary photosynthetic changes have been reported (Gonzalez *et al.*, 1996). The observed growth reductions were linked to UV-B-elicited decreases in cell division and cell expansion, resulting in decreases in leaf size and leaf number, thus reducing total leaf area and thereby photosynthesis at the whole plant level. Together with fewer and shorter stolons this ultimately resulted in lower white clover yield. The effects on cell division and expansion could be due to UV-B effects on plant growth substances. For example, the auxin indole acetic acid (IAA) can undergo photodestruction by UV-B (Huang *et al.*, 1997). Furthermore, flavonol compounds such as quercetin can decrease IAA activity or concentration, and increased flavonol levels under UV-B could thus affect IAA function also indirectly (Meijkamp *et al.*, 1999). While higher constitutive levels of quercetin glycosides could be related to slower growth in the stress-tolerant white clover ecotypes, these could in turn be better adapted to increased accumulation of these pigments under UV-B.

A UV-B-induced drop in available carbohydrate levels was observed both in 'Huia' and 'Tienshan'. However, significant UV-B-generated decreases in growth only occurred in 'Huia'. It also became obvious that decreased sugar levels did not lead to increased starch accumulation in the leaves or to higher foliar fibre content. This suggests that the available sugars could have at least partly been converted in other carbon-requiring metabolic functions. In addition to flavonoids resulting from aromatic amino acids originating in the shikimic acid pathway, UV-B stimulated accumulation of other amino acid types, including the heterocyclic proline and cyanogenic compounds based on the aliphatic valine and isoleucine. It became apparent that UV-B effects on such processes differ among white clover populations. From the correlation studies with UV-B responsiveness it can be suggested that white clover populations capable of specifically directing carbon flow towards flavonol, and particularly quercetin glycoside

accumulation, are better protected against UV-B than those directing these resources towards cyanogenesis or free proline accumulation. This suggests merit for carbon balance analyses in future UV-B studies. Both proline and the cyanogenic glycosides present in white clover are not likely to contribute to UV-B absorbance (Nahrstedt, 1981). Specific UV-B-induced shifts of carbon-containing substrates from primary metabolism to UV-B-protective secondary product formation have recently been described in parsley (Logemann *et al.*, 2000). The present work indicates a particular role for metabolic steps linking sugar and flavonoid metabolism in the protection of white clover against UV-B. Future studies should thus especially focus on the shikimate pathway, linking these two metabolic processes.

Within the flavonols, however, other issues than carbon balances also appear involved due to the highly specific increase in quercetin glycosides, relative to kaempferol compounds. In particular this indicates a crucial role for the enzyme flavonol 3' hydroxylase (F3'H), catalysing the hydroxylation of kaempferol to quercetin. Such pivotal involvement of F3'H has been suggested previously (Ryan *et al.*, 1998) and is further supported by the present work as no other possible alternative flavonoid compounds indirectly resulting from F3'H activity (e.g. myricetin via flavonol 3',5'-hydroxylase) were detected in the present studies. Specificity for UV-B-elicited *ortho*-dihydroxylated flavonoid accumulation has also been highlighted by other observations demonstrating that biosynthetically related compounds possessing 3',4'-dioxygenation, but excluding dihydroxylation, do not increase under UV-B (Markham *et al.*, 1998b).

It would appear that quercetin accumulation under UV-B in populations with moderate flavonol accumulation but highly efficient Q:K ratios ('Syrian', 'Prestige' and 'Kopu' - see Fig. 6-4) could largely depend on substrate availability. Assuming a biosynthetic relationship to flavonol levels, quercetin glycoside accumulation in these populations seems more limited by inefficiencies in earlier steps of flavonol synthesis, rather than also by inefficient quercetin conversion (as in 'Octoploid' and 'Haifa', Fig. 6-4). Other studies also report a central role for the dihydroxylated:monohydroxylated flavonoid ratio in circumstances of limited flavonoid accumulation under long-term UV-B irradiation (Markham *et al.*, 1998a; Ryan *et al.*, 1998). From the Q:K ratios it appears that the high UV-B-induced quercetin glycoside levels in ecotypic populations are a result of relatively efficient quercetin conversion from high flavonol levels while the

more productivity-oriented populations all are disadvantaged in their quercetin compound accumulation due to deficiencies in early, and some additionally in late, steps of quercetin synthesis. This suggests that these populations could be useful for detailed molecular examinations in several stages of flavonoid synthesis.

As mentioned briefly (Chapter 6), in addition to UV-B screening and potential antioxidant activities these flavonols could perform their functions also by harmless energy dissipation. Studies on keto-enol tautomerism point at UV-B protection by flavonols via excited state intramolecular proton transfer (ESIPT) (Smith and Markham, 1998). Absorbed UV energy could, via flavonol excitation, facilitate ESIPT between flavonol tautomers, for instance proton transfer from the excited state of an enolic tautomer to the keto form of the ground state, thus harmlessly dissipating potentially damaging UV-B radiation. Both quercetin and kaempferol glucosides have shown such keto-enol tautomerism, which is seen to be facilitated by the formation of flavonol dimers, especially at higher aqueous concentrations (Smith and Markham, 1998). This would be especially relevant in regard to the often very pronounced UV-B-induced increases of flavonoid levels, particularly flavonols, as observed in the present study. Furthermore, it has been shown that kaempferol-7-O-glucoside can also differ from quercetin-7-O-glucoside in aqueous solution (Smith and Markham, 1998). Depending on concentration, the kaempferol glucoside keto tautomer can be present at lower quantities in the ground state, with the majority of tautomers in the enol form. The enol tautomer absorbs in the blue spectral region while the absorption spectrum of the keto form is located in the UV region. Thus it is possible that quercetin glucoside, having relatively higher availability of the keto tautomer, could absorb and protect from UV-B more effectively compared to kaempferol glucoside (Smith and Markham, 1998). This suggests closer examination of energy dissipation roles for such compounds in future UV-B studies.

Such studies should also explore the population-dependent accumulation of UV-B-induced flavonoids, especially quercetin and kaempferol glycoside production, steady state and degradation. Further work needs to establish whether marked increases in these flavonol compounds are systemic. Pronounced systemic flavonol increases could, in addition to effects on plant morphology and physiology, also impact on ecosystem processes. The observed links between quercetin glycoside accumulation and biomass

production warrant detailed investigations of possible linkages to growth regulatory mechanisms, e.g. effects on auxin function. The synergistic interaction of UV-B and drought for the initiation of these compounds clearly warrant such investigations in combination with other forms of stress. Optical studies could determine whether UV-B penetration through the white clover epidermis, particularly directly underneath central cell regions, differs among white clover populations. This, in conjunction with measurements of UV-attenuating pigmentation in the epidermal layer would assist in the verification of differential epidermal cell shape contributing to UV-B protection.

The findings of this work also suggest detailed investigations on UV-B effects on plant growth substances in white clover. Decreases in processes linked to cell division in plant parts shielded against UV-B indicate indirect, rather than direct UV-B effects on cell division. The results could suggest a particular focus on IAA function, but from the observations on reduced cell division also on cytokinins, gibberellins and other growth regulators. The observed UV-B effect on young leaf and bud growth could affect both auxins and gibberellins, which are both produced in these organs. This could also help explain the observed decreases in shoot elongation. Cytokinins can decrease under drought stress (Shashidhar *et al.*, 1996) and have been shown to counteract negative UV-B effects (Jain *et al.*, 1988). Abscisic acid levels increase in drought (Shashidhar *et al.*, 1996) and under UV-B (Rakitina *et al.*, 1994). Combined investigations of UV-B with drought on this growth inhibitor could be of particular interest in consideration of the marked improvements in leaf water status under UV-B observed here. Decreases in elongation growth could also be related to increases in ethylene production, a hormonal response that has been observed under UV-B (Predieri *et al.*, 1993). From other studies it is possible that the observed population-specific UV-B effects in white clover are related to differential efficiency of jasmonic acid and ethylene signal pathways (Mackerness *et al.*, 1999).

Organ-specific studies

The main focus of this research was on white clover leaf attributes. This is both due to the primary role for UV-B reception by these organs and because of their predominant contribution to white clover yield. However, findings from this work also suggest merit

for future studies focussing on other plant organs in white clover. These experiments did not aim at a comprehensive investigation of other polyphenolics, e.g. lignin or tannins. Lignin is part of NDF (neutral detergent fibre) which showed decreased levels in 'Huia' and no change in 'Tienshan' (Fig. 7-1). Condensed tannins only occur at negligible quantities in white clover leaves (Caradus *et al.*, 1995). It would however be tempting to investigate whether UV-B affects levels in plant organs that contain higher amounts of these polyphenolics, i.e. in stolons for lignin and in flowers for tannins. The latter would also be of particular relevance in field studies as condensed tannins increase protein utilisation and reduce bloat in ruminants (Dakora, 1995).

Visitation by pollinators can be changed by UV-B-induced changes to plant characteristics of importance for attraction of beneficial insects (Conner and Zangori, 1997). Reproductive parts of the plant like pollen and ovules are relatively well protected from UV-B. Anther walls, for instance can absorb more than 98% UV-B (Flint and Caldwell, 1984). A susceptible target to UV-B, however, is the germinating pollen after transfer to the stigma. UV-B damage can alter *in vitro* pollen germination (Flint and Caldwell, 1984). This would be of particular relevance in consideration of the observed changes in flavonoid compounds which are involved in the processes of pollinator attraction and germination (Dakora, 1995).

Taking into account the lower soluble sugar and starch levels in white clover leaves under UV-B (Fig. 7-1) it would be interesting to examine whether starch levels would increase in the roots. Given higher root:shoot ratios under UV-B (Table 2-2), it is possible that some carbon was preferentially exported towards roots. This could again suggest mediation of the UV-B response via plant growth substances. However, heterogeneity of damage between aerial and belowground plant organs can also be due to organ-specific differences at the molecular level. In maize, less DNA damage was observed in roots compared to leaf tissues and this was related to either physical separation from UV-B exposure or higher rates of DNA repair (Stapleton *et al.*, 1997). Increased relative root mass also suggests examination of other important belowground white clover functions, including nodulation and interaction with beneficial microbes. A relative improvement of root development could in the longer-term benefit the shoot due to possible increases in total nodulation of the larger root surface.

This is also of particular relevance in regard to the observed increases in flavonols as they are involved in beneficial interactions with soil microbes (Dakora, 1995). Production of flavonoids which are released by legume roots and which are involved in the inoculation of the plant by symbiotic nitrogen-fixing bacteria can be affected by UV-B (Sallaud *et al.*, 1995). Treatment of three tropical leguminous crops with elevated UV-B resulted in reduced nitrogen fixation via decreased nodule activity, accompanied by alterations in growth and net photosynthetic rates (Singh, 1997). Alterations of symbiotic interactions through the influence of UV-B radiation could have wide-ranging implications for the New Zealand pasture ecosystem, largely reliant on such relationships. The cultivar 'Huia' belongs to a group of white clover populations that is particularly dependent on its nitrogen supply through symbiosis with nitrogen fixing bacteria, especially under conditions of low soil nitrogen. Effects on this interaction could again lead to effects of UV-B on population dynamics within New Zealand pasture ecosystems.

Population-specific studies

As mentioned above, these studies revealed a number of constitutive and UV-B-induced differences among the white clover populations. The background information on the environmental and breeding background of all white clover populations provided here will also be useful for future classifications of UV-B responsiveness based on plant functional types. Apart from the general distinctions in UV-B responsiveness between stress-tolerant ecotypes and productive cultivars, specific issues were also raised from observations on individual white clover populations. For example, productivity in some high-yielding white clover populations (e.g. in 'Octoploid' and 'Kopu', Fig. 3-1a,b) still exceeded that of the UV-B-tolerant ecotypes, even when reduced by UV-B exposure. Thus the absolute yield in some white clover population could still be sufficient even though they are more UV-B sensitive. Long-term studies on higher-yielding white clover populations should examine whether this would be maintained during growth conditions in the field. Such studies should also take into consideration possible shifts in UV-B sensitivity for some populations. Compared to the short-term screening experiment, the cultivar 'Prestige' in particular appeared more UV-B-sensitive during growth in the longer-term UV-B study (Fig. 9-3). Thus longer-term UV-B exposure

could have overcome initial UV-B tolerance in this cultivar which might be related to some UV-B adaptation in its habitat of origin (Table 3-1). UV-B tolerance in 'Luclair' should be further explored, also in regard to the optical measurements mentioned above. It would be tempting to investigate the possible sunscreen role for translucent 'Luclair' leaves by measuring UV-B transmission below the clear leaf regions. UV-B tolerance in 'Syrian' was accompanied by relatively low flavonol accumulation. 'Syrian' was selected for tolerance against leaf rust and such tolerance has been related to higher constitutive polyamine levels (Bharti and Sawhney, 1996) as well as enhanced oxidase activity, including peroxidases (Sharma and Sharma, 1997). Peroxidases and polyamines have been implicated in the possible alleviation of UV-B-induced oxidative stress (Kramer *et al.*, 1991; Tekchandani and Guruprasad, 1998). Specific selection of 'Syrian' could have led to the preference for one UV-B-related antioxidant system at the cost of another.

Interaction of UV-B with additional stress, disturbance and competition

From the stress interaction studies in this thesis it can be suggested that white clover growth may be less affected by UV-B during periods of drought, compared to moist seasonal conditions. However, it appears that a possible beneficial interaction may only come to effect during more extended drought periods. Climate change projections of enhanced UV-B irradiation effects will increasingly need to take into account alterations in patterns and amounts of moisture supply. The observed UV-B-induced decreases in leaf area as well as increases in proline levels, leaf water status, root:shoot ratio and stomatal density could have beneficial effects for subsequent stages of recovery from drought.

Considering its usual association with ryegrass in the New Zealand pasture ecosystems - and the relative insensitivity of ryegrass to UV-B observed in concomitant studies (Campbell and Hofmann, 1998) - competitive relationships could also be affected by UV-B, and these might in turn be further modified in dependency to water availability (Chapter 1). The findings also indicate a need for future studies examining interaction of UV-B with defoliation, particularly in pasture plants such as white clover that are regularly subjected to grazing under field conditions. Furthermore the results from bioassay studies indicate that UV-B herbivory studies should examine genotypic effects

in both plants and animals. Close scrutiny should be given to potential impacts on herbivory via UV-B-induced increases in secondary metabolites, including cyanogenesis. Long-term studies would be of value to test possible cumulative effects on herbivory and reproductive success over several insect generations. As concerns the observed time dependency of the UV-B response under continuous UV-B exposure it would be important to test whether this interaction undergoes modifications during the daily and seasonal UV-B fluctuations in the field.

Should marked UV-B-induced biochemical changes on major plant nutrients also occur in the field, then this could potentially impact on mammalian herbivores such as larger ruminants normally feeding on white clover. Decreased carbohydrate levels could affect white clover palatability and suitability as silage. The high levels of nitrogen present naturally in white clover are known to lead to inefficiencies in the utilisation of herbage protein in ruminants (Wanjaiya *et al.*, 1993) and a further UV-B-induced increase could enhance this effect. Furthermore, an increase in nitrogen levels could lead to lower utilisation of NDF by ruminants (Wanjaiya *et al.*, 1993), potentially intensifying effects from UV-B-induced decreases in 'Huia' NDF levels observed here.

The cyanogenesis levels in 'Huia' can be classified as moderate-high but still ranging below levels considered as critical for ruminant grazing (Caradus and Woodfield, 1997; Crush and Caradus, 1995). Marked UV-B-induced increases of cyanogenesis will have to be investigated as there is a potential that sufficiently high levels in white clover diets could cause cyanide poisoning and nutrient deficiency in ruminants. Possible consequences could include decreased wool production in sheep, goitrogeny and nutritional myopathy (Caradus *et al.*, 1995; Gutzwiller, 1993; Lehmann *et al.*, 1991). Should potential increases in cyanogenesis under UV-B remain below the sensitivity thresholds for mammalian herbivores, there may however still be beneficial consequences via possible insecticidal cyanogenic effects.

Taken together these findings point to a number of avenues for future research, from metabolic processes linked to specific flavonoid synthesis to the population biology of UV-B effects and resulting impacts on pasture ecosystems.

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