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Independent responses to ultraviolet radiation and herbivore attack in broccoli

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Abstract

The plant responses to ultraviolet-B radiation (UV-B) and to insect herbivory are believed to be partially similar. In this study, responses to these factors were investigated in the crop species broccoli (*Brassica oleracea* L. convar. *botrytis*, Brassicaceae). Plants were first grown under three UV-B regimes (80%, 23%, and 4% transmittance of ambient UV-B) in greenhouses covered with either innovative materials (high and medium transmittance) or conventional glass (low transmittance). Half of the plants then remained under these conditions, but the other half were transferred to the field with ambient light and herbivore access for up to 3 d. The plant responses to distinct environmental conditions were examined by analysing the morphological and chemical parameters of plants kept inside and plants exposed in the field. Furthermore, suitability of field-exposed plants to naturally occurring insects was investigated in relation to UV-B pretreatment. High levels of UV-B radiation led to increased flavonoid concentrations, but to a lower biomass accumulation in broccoli. These patterns remained after outdoor exposure. However, UV-induced changes of plant traits did not alter attractiveness to herbivorous insects: thrips, whiteflies, and aphids attacked plants independently of UV-B pretreatment. A 3-fold increase of indolyl glucosinolate concentrations occurred in above-ground tissue of all the plants, most likely due to massive herbivore attack after 3 d of field exposure. The results show that plants respond with high specificity to different abiotic and biotic impacts, demonstrating the separate perception and processing of stress factors.

Key words: Biomass, Brassicaceae, flavonoids, glucosinolates, herbivory, metabolite induction, plant–insect interactions, plant responses, UV-B radiation.

Introduction

Plants are sessile organisms that are exposed to various abiotic and biotic environmental impacts. Plant responses to different environmental stresses such as ultraviolet UV-B (UV-B; 280–315 nm) and herbivory can overlap, measurable, for example, as the gene expression pattern of signalling pathways (Izaguirre *et al.* 2003; Stratmann, 2003). However, plants should have stress-specific mechanisms to adjust to these multi-faceted environmental impacts (Lichtenthaler, 1998; Jansen *et al.*, 2008).

Sunlight is an important abiotic factor that influences various developmental processes in plants. A highly dynamic and energy-rich fraction of the solar spectrum that

reaches the earth's surface is UV-B radiation (Paul and Gwynn-Jones, 2003). Depending on the physiological and developmental status of the plant and on the quality and duration of the UV-B exposure, this radiation can cause damage to macromolecules, generate reactive oxygen species, and act as an environmental stressor (Rozema *et al.*, 1997; Jansen *et al.*, 1998; Jenkins and Brown, 2007). UV-B can also function as a signal stimulating developmental processes of plants and promoting plant survival (Ulm and Nagy, 2005; Jenkins and Brown, 2007; Brown and Jenkins, 2008; Safrany *et al.*, 2008). Due to their high plasticity, plants respond with characteristic phenotypic acclimation

processes to UV-B such as reduced growth and/or an increased accumulation of phenolic compounds, which act in epidermal cells as a sunscreen (Caldwell *et al.*, 2007).

Little is known about UV-induced signalling processes. UV-B-mediated specific photomorphogenetic signalling is distinct from non-specific stress responses (Jenkins and Brown, 2007). However, UV-B-induced signalling pathways and wound-responsive signalling partly overlap (Izaguirre *et al.*, 2003; Stratmann, 2003). In response to UV-B-mediated plant changes, reduced insect herbivory on UV-B-irradiated plants compared to non-UV-B-irradiated plants, has been observed in several plant–insect systems (Ballaré *et al.*, 1996; Rousseaux *et al.*, 1998, 2004; Caputo *et al.*, 2006). In addition to these plant-mediated effects, direct effects of UV radiation on insect behaviour can influence plant–insect interactions (Antignus *et al.*, 1996; Costa and Robb, 1999; Kuhlmann and Müller, 2009; Mazza *et al.*, 1999, 2002).

Apart from phenolic metabolites such as flavonoids, which serve as UV-protection, plants produce specific compounds to prevent damage by herbivorous insects. Glucosinolates are the characteristic defence-related secondary metabolites of the Brassicaceae. Both flavonoid and glucosinolate induction depend partly on the same signalling pathways, which involve jasmonic acid (A-H-Mackerness *et al.*, 1999; Textor and Gershenzon, 2009). Glucosinolates are nitrogen- and sulphur-containing metabolites, which are known for their deterrent effects on generalist herbivores, whereas they may stimulate feeding and oviposition by specialists (Halkier and Gershenzon, 2006). Upon insect feeding damage, Brassicaceae often respond with an increase in glucosinolate concentrations (Hopkins *et al.*, 2009; Textor and Gershenzon, 2009).

For several Brassicaceae species, responses to UV-exposure as well as influences on herbivores have been studied (Grant-Petersson and Renwick, 1996; Caputo *et al.*, 2006; Foggo *et al.*, 2007). Broccoli plants (*Brassica oleracea* L. convar. *botrytis*) exposed to ambient UV-A (315–400 nm) and UV-B levels, or grown under reduced levels of UV radiation, only show differences in biomass accumulation when plants experience the different environmental conditions during germination and early growth. Plants germinated under ambient UV radiation levels are smaller. By contrast, plants grown under low-UV and subsequently transferred to different UV-conditions are not affected in growth (Kuhlmann and Müller, 2009). Leaf flavonoid concentrations increase when UV-A and UV-B irradiance has been higher in all Brassicaceae species investigated to date (Reifenrath and Müller, 2007; Kuhlmann and Müller, 2009). By contrast, glucosinolate levels, as well as proteinase inhibitor activities, are unaffected by different irradiance (Reifenrath and Müller, 2007, 2008; Kuhlmann and Müller, 2009), indicating an independent regulation of different defence metabolites. However, plant responses to herbivory in relation to UV-B treatment conditions were not considered in these studies.

Vegetables such as broccoli usually germinate in greenhouses and are transplanted after a few weeks to the field.

At this point plants are not adapted to ambient radiation conditions, because conventional greenhouse glass has zero or low UV-B transmittance. In this study, the effects of UV-B treatment on young broccoli plants grown in greenhouses, as well as plant responses after the transfer to common field conditions with herbivore access, were investigated. For germination and early growth, plants were placed in three differently covered greenhouses, of which two were covered with innovative materials. The cover materials transmitted high, medium (innovative materials) or low levels (conventional glass) of ambient UV-B radiation but had almost equally high levels of UV-A transmittance (Table 1). Plant biomass as well as defence metabolites (flavonoid and glucosinolate concentrations) and nutritional state (carbon:nitrogen ratio, C/N) were measured from greenhouse-kept plants and from plants that were transferred to the field after different UV-B pretreatments for up to 3 d. Furthermore, the suitability of outdoor-transferred plants to naturally occurring herbivorous insects and the insects' impact on plant chemistry were examined in relation to UV-B pretreatment. It was expected that the plants' defence response to UV and putative herbivores would depend on the radiation quality that the plants had experienced during the early growing period in the greenhouses. High-UV-B pretreated plants may be less suitable for herbivores than plants that are only exposed to low UV-B at an early growth stage due to the overlapping defence responses that modify the abundance of secondary metabolites. Finally, effects on plant chemistry due to herbivore feeding were expected.

Material and methods

Plants and design of the experiment

Broccoli plants [*Brassica oleracea* L. convar. *botrytis* (L.) Alef. var. *cymosa* Duch. Monopoly F_1 hybrid; Syngenta Enkhuizen, Netherlands] ($n=150$) were grown from seeds in three differently covered greenhouses ('UV-B treatment') from which insects were excluded (for greenhouse construction see below; average temperature 26 °C, average humidity 60%). Plants were grown in fertilized soil (ED 73, pH 6) in individual pots (diameter, 12 cm, height, 9 cm).

Seventeen days after sowing (plants at the four leaf-stage, 0 h), the above-ground tissue of ten plants from each

Table 1. Transmittance (%) of greenhouse covering materials

Fractions of sunlight are classified to UV-B (280–315 nm), UV-A (315–400 nm), and PAR (photosynthetic active radiation, 400–700 nm).

Transmittance	UV-B treatment		
	High UV-B	**Medium UV-B**	**Low UV-B**
UV-B (%)	80	23	4
UV-A (%)	87	84	75
PAR (%)	97	95	92

greenhouse was harvested for biomass determination and chemical analysis. Half of the remaining plants were kept in the greenhouses ($n=60$), the other half ($n=60$) were transferred outdoors ('exposure treatment'). Outdoors, plants were randomly positioned on a flat surface in the field, which was covered by a mulch film. The field was 10 m from the greenhouses. Plants were located in two rows with a distance of 30 cm between one another. To evaluate changes over time, ten plants from each condition (greenhouse-kept and outdoor plants of different UV-B radiation (pre-)treatments) were harvested for biomass determination as well as chemical analysis at day 18 (24 h after the first harvest and outdoor exposition, respectively) and at day 20 (72 h afterwards). Outdoor plants were inspected for insect infestation (see below). During the outdoor exposure time of broccoli, the mean temperature was 17 °C. During the entire growth and experimental period (16 May to 4 June 2007) ambient radiation levels averaged 20 kJ m^{-2} d^{-1} UV-B, 1390 kJ m^{-2} d^{-1} UV-A, 6250 kJ m^{-2} d^{-1} PAR (photosynthetic active radiation) and 18 170 kJ m^{-2} d^{-1} global radiation [recorded by a meteorological station (Thies Clima, Göttingen, Germany) located 25 m from the greenhouses]. During the outdoor exposure period radiation averaged 20 kJ m^{-2} d^{-1} UV-B, 1465 kJ m^{-2} d^{-1} UV-A, 6270 kJ m^{-2} d^{-1} PAR and 18 500 kJ m^{-2} d^{-1} global radiation.

Construction of greenhouses

Broccoli plants were grown in three experimental greenhouses in the Botanical Garden of Würzburg. These greenhouses had a ground area of 4.2 m×3.0 m and were covered with different materials, which transmitted distinct ranges of UV-B radiation. Transmittance measurements of greenhouse materials were conducted under a cloudless sky at noon with an $X1_2$ Optometer (Gigahertz Optik, Puchheim, Germany; Table 1). The longer axis of each greenhouse was aligned in a north–south direction. The roof was subdivided into three parts and sloped from 3.9 m in height (north) to 2.0 m in height (south). There were three inclinations of the roof from north to south of 14°, 21.8°, and 28.8°. Plants were placed on U-shaped tables (85 cm in height) joined to the east, south, and west walls of the greenhouses (design of the greenhouses by Gerhard Reisinger, University of Bonn, Germany, construction by Siedenburger Gewächshausbau, Radhen, Germany). One greenhouse was covered with conventional float glass (low UV-B transmission; Siedenburger Gewächshausbau, Radhen, Germany), the second with CENTROSOL MM solar glass, which was micro-structured on both sides (medium UV-B transmission; Centrosolar Glas, Fürth, Germany), and the third was covered with ethylene-tetrafluorethylene (ETFE) foil (high UV-B transmission; Asahi Glass Green-Tech, USA, China, South Korea, Japan) (Table 1). Only one greenhouse of each type could be built due to the high costs. The greenhouses were closed systems to prevent insect access. Air circulation was provided by ventilators (Univent Ventilatoren, Villingen-Schwenningen, Germany). Evaporative cooling was achieved by spraying two woven acryl fabric tubes (Schumann, Energieschirm und Schattierungstechnik, Kleinmaischeid, Germany) per house with water when the temperature of the houses exceeded a defined threshold of 23 °C. These were arranged under the east and west arms of the U-shaped tables.

Biomass determination and chemical analyses

The harvested above-ground broccoli plant material was immediately frozen in liquid nitrogen, stored at –80 °C and lyophilized to prevent any enzymatic degradation. Dry weight was determined. For chemical analyses, the lyophilized material was homogenized (mixer mill 301, Retsch, Haan, Germany). The carbon and nitrogen contents were measured by quantitative decomposition of substances by oxidative combustion (CHN-O-Rapid, Elementar, Hanau, Germany).

For the determination of flavonoid aglycones, samples were hydrolysed according to protocols modified from Kolb *et al.* (2001) and Vallejo *et al.* (2004). Aliquots of dried plant material were extracted in aqueous 80% methanol with the flavonol myricetin (Fluka, Seelze, Germany) as internal standard, and extracts were dried. Dried extracts were redissolved in aqueous 80% methanol, and hydrolysed after the addition of an equal volume of 2.5 M HCl for 30 min at 85 °C. Hydrolysis was stopped on ice and diethyl ether was added for phase separation. The upper diethyl ether fraction was taken, dried, and dissolved in 80% aqueous methanol. These extracts were analysed by HPLC (1100 Series, Hewlett-Packard, Waldbronn, Germany) with a quaternary pump and a 1040M diode array detector. Gradient separation of flavonoid aglycones was achieved on an Agilent Zorbax Bonus RP column (250 mm×4.6 mm×5 μm) with an eluent gradient (solvent A: 0.5% acetic acid in purified water, solvent B: acetonitrile) of 5–50% B (5 min), 50% B (5 min hold), 50–95% B (5 min), 95% B (5 min hold) followed by a cleaning cycle. Flavonol aglycones were identified by a comparison of retention time and UV spectra with those of purified standards (standards from Phytoplan, Heidelberg, Germany and Extrasynthese, Genay, France). Quantification was achieved by integration of the peak area at 360 nm (bandwidth 4 nm) relative to the area of the internal standard peak, corrected by response factors (0.79 for quercetin, 0.75 for kaempferol, determined by repeated injection of known concentrations of the reference samples).

For the determination of glucosinolate concentrations, dried plant material was extracted in aqueous 80% methanol with benzyl glucosinolate (Phytoplan, Heidelberg, Germany) as an internal standard. Glucosinolates were converted to desulphoglucosinolates using purified sulphatase (EC 3.1.6.1, 'type H-1, from *Helix pomatia*, 15 100 units g^{-1} solid; Sigma, Taufkirchen, Germany; purified after Graser *et al.*, 2001). The desulphoglucosinolates were analysed by HPLC, identified, and quantified as previously described by Müller and Wittstock (2005) and Gigolashvili *et al.* (2007).

Insect infestation of outdoor exposed plants

Infestation of plants by naturally occurring insects was recorded by counting the number of infested and uninfested plants (24 h) and by counting all insects per plant (72 h) on the entire above-ground biomass of broccoli plants placed outdoors. At the second time point (72 h) all plants were already heavily infested by insects. Insects were determined to family level (Aleyrodidae, Aphididae, and Thripidae).

Statistical analysis

Individual potted plants of the three greenhouses were considered as true replicates. Biomass and chemical parameters of 17-d-old plants of the 'UV-B treatment' group were compared using one-way ANOVA (G 0 h). Parameters of plants from the 'exposure treatment' that remained either in the greenhouses (G 72 h) or were exposed outdoors for 72 h (F 72 h) were compared by MANOVA. Plant parameters were transformed where necessary to reach homogeneity of variances. The proportion of insect-infested plants versus uninfested plants after 1 d exposure was evaluated by Pearsons Chi^2. The number of insects per plant and per gram fresh weight, respectively, were analysed after exposure for 3 d using Kruskal–Wallis analysis of ranks. To calculate the relationship of different chemical parameters and the number of insects per plant after outdoor exposure (72 h), Spearman rank correlations were performed. Data analysis was performed with Statistica 8.0 (StatSoft, Tulsa, USA).

Results

Broccoli plants grown under different UV-B regimes showed treatment-dependent responses. Above-ground biomass accumulation was highest for plants grown under low UV-B conditions and lowest for plants grown under high UV-B irradiation ('UV-B treatment', 0 h; Table 2; Fig. 1). This UV-B related difference in biomass accumulation persisted after 72 h of common field exposure. Furthermore, biomass was significantly higher in greenhouse-kept plants compared to field-exposed plants after 72 h, probably due to the warmer temperatures in the greenhouses ('exposure treatment', 72 h; Table 2; Fig. 1).

The C/N ratio of plants grown under high UV-B was significantly lower than that of plants grown under low UV-B greenhouse conditions ('UV-B treatment', 0 h; Table 2; Fig. 1). After 72 h of field exposure, the C/N ratio of all outside exposed plants dropped significantly ('exposure treatment', 72 h; Table 2; Fig. 1) due to a relative increase in nitrogen per dry weight compared to greenhouse-kept plants. The total nitrogen content per plant was not significantly different between greenhouse-kept and field-exposed plants ('exposure treatment', 72 h, MANOVA for total nitrogen content per plant: $F_{(2;51)}=0.026$; $P=0.873$).

Plants grown under high UV-B conditions had higher quercetin and kaempferol concentrations ('UV-B treatment', 0 h; Table 2; Fig. 1) as well as a significantly higher quercetin/kaempferol ratio than medium and low UV-B exposed greenhouse plants ('UV-B treatment', 0 h; one-way ANOVA for quercetin/kaempferol ratio: $F_{(2;27)}=28.60$; $P<0.001$). Differences in quercetin and kaempferol flavonoid concentration were still present after 72 h field exposure, with plants taken from the high UV-B treatment showing significantly higher flavonoid concentrations than plants from the other two treatments ('UV-B treatment', 72 h; Table 2; Fig. 1). All plants exposed outdoors had significantly increased quercetin concentrations compared with greenhouse-kept plants after 72 h, whereas kaempferol concentrations did not change significantly ('exposure treatment', 72 h; Table 2; Fig. 1). The differences in the quercetin/kaempferol ratio diminished after 72 h of field

Table 2. Impact of 'UV-B treatment' (0 h, 72 h) and 'exposure treatment' (72 h) on growth and chemical parameters of above-ground tissue of broccoli plants

Treatment effects were analysed by one-way ANOVA (0 h) and MANOVA (72 h). Plants were grown in greenhouses with different levels of UV-B irradiation ('UV-B treatment', 80%, 23%, and 4% UV-B transmittance). After 17 d, half of the plants from each UV-B condition were kept in the greenhouses, whereas the other half were exposed outdoors for 72 h ('exposure treatment'). GS, glucosinolates; DW, dry weight. Boldface indicates $P<0.05$. Asterisks denote significant P-values after Bonferroni correction carried out following Benjamini and Hochberg (1995) for each time of harvest. Some plant parameters were transformed to reach homogeneity of variances [0 h: quercetin ($x^{1/2}$); 72 h: C/N ratio ($1\ x^{-5}$), indolyl GS ($x^{1/3}$), kaempferol (log x)]. Measured data are shown in Fig. 1.

| Plant parameters	0 h ANOVA		72 h MANOVA					
	UV-B treatment		UV-B treatment		Exposure treatment		UV-B treatment× exposure treatment	
	$F_{(2;27)}$	P	$F_{(2;53)}$	P	$F_{(1;53)}$	P	$F_{(2;53)}$	P
Dry weight (g)	11.09	**<0.001***	24.02	**<0.001***	7.20	**0.010***	0.94	0.396
C/N ratio	3.38	**0.049**	1.15	0.324	50.23	**<0.001***	0.98	0.384
Quercetins (µmol g^{-1} DW)	184.48	**<0.001***	106.40	**<0.001***	438.60	**<0.001***	2.71	0.076
Kaempferols (µmol g^{-1} DW)	72.58	**<0.001***	63.17	**<0.001***	1.09	0.301	1.57	0.218
Aliphatic GS (µmol g^{-1} DW)	7.87	**0.002***	2.07	0.137	22.74	**<0.001***	3.34	**0.043**
Indolyl GS (µmol g^{-1} DW)	2.28	0.122	0.27	0.761	261.99	**<0.001***	3.27	**0.046**
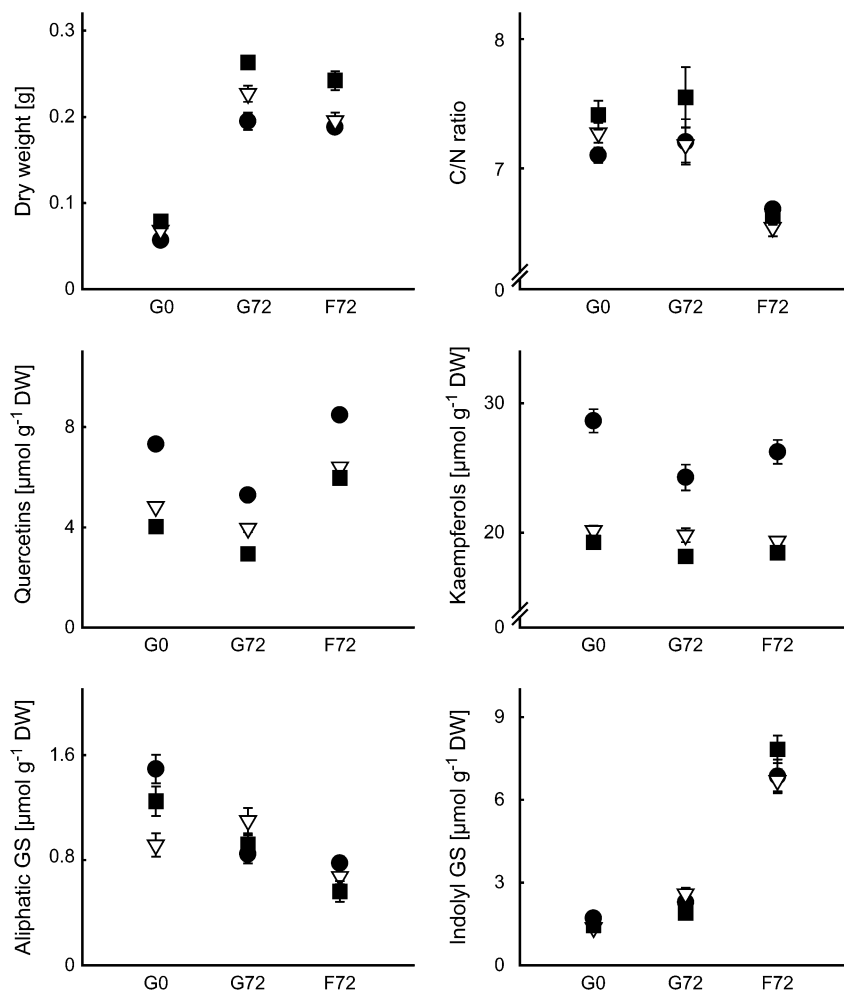
Fig. 1. Plant parameters (mean ±SE, n=10) of broccoli above-ground tissue grown under different UV-B irradiance and exposure conditions. Plants were grown in greenhouses for 17 d (G 0) with different levels of UV-B irradiation (80%, 23%, and 4% transmittance). After 17 d, half of the plants from each condition were exposed outdoors for 72 h (F 72), the other half remained in the greenhouses (G 72). For statistical analyses see Table 2. Please note the different scales of the y-axes. GS, glucosinolates; DW, dry weight. Filled circles, high UV-B, open triangles, medium UV-B, filled squares, low UV-B.

exposure, probably due to a higher relative increase of quercetin flavonols in medium- and low-UV-B pretreated plants compared with high-UV-B pretreated plants ('UV-B treatment', F 72 h; one-way ANOVA of outdoor-exposed plants from different pretreatments: $F_{(2;27)}$=0.33; P=0.725). In general, kaempferol concentrations were over three times higher than quercetin concentrations in all plants (Fig. 1).

In this experiment, only one greenhouse of each type could be used, therefore one might consider the individual potted plants only as pseudoreplicates. However, in repeated experiments broccoli always responded in a similar fashion with reduced growth and increased flavonoid concentrations when grown under high UV-B conditions (F Kuhlmann, unpublished results).

The 'UV-B treatment' had a significant effect on the concentration of aliphatic glucosinolates but was not directly related to the UV-B radiation levels plants received in the greenhouses (0 h; Table 2). Plants of the medium-UV-B treatment showed the lowest concentrations of aliphatic glucosinolates (Fig. 1). After outdoor exposure, no consis-

tent effects could be detected for these metabolites. Overall, aliphatic glucosinolate concentrations were low (72 h; Fig. 1). Concentrations of indolyl glucosinolates were unaffected by 'UV-B treatment' (0 h; Table 2; Fig. 1). By contrast, the 'exposure treatment' had a significant effect on glucosinolate accumulation (72 h; Table 2). Field exposure of plants resulted in a 3-fold induction of indolyl glucosinolates after 72 h, up to about 8 μmol g^{-1} dry weight (Fig. 1). After 24 h field exposure, the total and indolyl glucosinolate concentrations were, on average, still low with 2.6 and 1.8 μmol g^{-1} dry weight, respectively (compare with glucosinolate concentrations in Fig. 1).

Plant reactions to 72 h outdoor exposure were independent of the pretreatment in the different greenhouses, except for aliphatic and indolyl glucosinolates, for which a significant interaction between 'UV-B treatment'×'exposure treatment' was detectable (72 h; Table 2; Fig. 1).

Plants grown under different UV-B regimes and exposed for 24 h or 72 h in the field were not significantly differently infested by insects (24 h; number of infested versus

uninfested plants: Pearsons Chi$^2_{(n=10)}$=4.29; P=0.117; 72 h; number of insects per plant and per unit biomass; Table 3). The plants were mainly infested by Thripidae.

After 72 h of outdoor exposure, total glucosinolate amount per plant was significantly positively correlated with the number of insects found on each plant ($R_{(n=29)}$=0.45; P=0.013), but a relationship between total flavonoid amount and total insect infestation per plant was not observed ($R_{(n=29)}$=0.05; P=0.979). Total glucosinolate concentrations did not correlate with total flavonoid concentrations ($R_{(n=29)}$=–0.24; P=0.216).

Discussion

Plants are able to recognize and respond to their surrounding environment with high specificity. Broccoli plants grown under different UV-B irradiance in greenhouses covered with innovative materials and later transferred to common field conditions with unrestricted herbivore access, showed increases in flavonoid concentrations and reduced growth, which were related to UV-B. Whereas these traits changed over time, the pattern (flavonoid concentration and growth differences between plants of different UV-B conditions) remained the same. By contrast, an increase of glucosinolate concentrations was induced, most likely by herbivore attack in the field. UV-B pretreatment did not influence the plant susceptibility and attractiveness to naturally occurring insect herbivores.

A protection against potentially detrimental UV-B radiation is generally achieved by the induction of phenolic compounds (Caldwell *et al.*, 1983). In accordance with this, the concentration of quercetin and kaempferol flavonols in broccoli plants was positively related to the irradiance the plants faced in the differently covered greenhouses. However, the production of UV-B-screening pigments is likely to involve some constraints, as the accumulation of biomass was reduced in plants confronted with higher UV-B irradiance (Table 2;

Table 3. Infestation of broccoli plants (mean ±SE number of insects per plant and per gram fresh weight, FW, n=10 plants) after 72 h of field exposure by Aleyrodidae, Aphididae, and Thripidae

Plants were grown in greenhouses with different levels of UV-B transmittance (80%, 23%, and 4%) before field exposure. Statistical analysis was performed using Kruskal–Wallis analysis of ranks.

Insect families	Plant pretreatment				
	High UV-B	Medium UV-B	Low UV-B	$H_{(2, n=30)}$	P
Aleyrodidae plant^{-1}	3.3±1.1	2.2±0.7	2.5±0.5	0.51	0.776
Aphididae plant^{-1}	0.2±0.1	3.5±2.0	2.5±1.1	4.63	0.099
Thripidae plant^{-1}	22.0±3.3	21.0±2.5	22.5±2.7	0.09	0.955
Aleyrodidae g^{-1} FW	2.2±0.7	1.4±0.5	1.3±0.4	0.76	0.684
Aphididae g^{-1} FW	0.1±0.1	2.6±1.5	1.0±0.6	2.67	0.264
Thripidae g^{-1} FW	14.7±2.1	13.1±1.2	12.4±1.4	0.87	0.649

Fig. 1). A negative relationship between biomass and flavonoid concentration has also been observed in earlier experiments with broccoli that received different irradiances of UV-A and UV-B from germination onwards (Kuhlmann and Müller, 2009). From the current results, it can be concluded that the increased flavonoid concentration at reduced growth is predominantly caused by UV-B, whereas UV-A plays a subordinate role. Smaller plants may have experienced reduced cell expansion due to higher UV-B irradiance as has been proposed by previous studies (Rozema *et al.*, 1997; Jansen *et al.*, 1998; Jansen, 2002; Hectors *et al.*, 2007). It remains to be seen if flavonoid induction and biomass reduction are directly or indirectly linked, and whether those phenotypic changes in plants are typical responses to UV-B. Morphogenetic changes in *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae) exposed to low dose rates of UV-B radiation without stress were interpreted as a redistribution rather than a cessation in plants (Hectors *et al.*, 2007).

Kaempferol and quercetin flavonols responded differently in broccoli plants. Although kaempferol flavonol concentrations were higher in all plants, the quercetin/kaempferol ratio was highest in high UV-B exposed plants (Fig. 1). A relatively higher increase of quercetin compared to kaempferol flavonol concentrations in UV-exposed plants has also been found in earlier studies (Markham *et al.*, 1998; Olsson *et al.*, 1998; Hofmann *et al.*, 2003; Reifenrath and Müller, 2007; Winter and Rostás, 2008). It has been suggested that quercetin flavonols have a better ability for free radical scavenging than kaempferol flavonols (Harborne and Williams, 2000).

Besides the flavonoid concentration, the C/N ratio was influenced by 'UV-B treatment'. However, after 72 h of outdoor exposure, differences in C/N ratio disappeared between differently pretreated plants and field-exposed plants had higher nitrogen concentrations than greenhouse-kept plants (Table 2; Fig. 1). This may be due to growth and allocation changes. Both flavonoid levels and C/N ratio are known to be important factors influencing herbivore nutrition (Harborne and Williams, 2000; Awmack and Leather, 2002; Treutter, 2005). Despite these UV-B-induced changes in plant quality no significant differences in insect infestation of field-exposed plants could be observed (Table 3). Thus, flavonoids primarily acted as a sunscreen but not as a defence against herbivores (Close and McArthur, 2002; Izaguirre *et al.*, 2007). Broccoli plants mainly attracted thrips, which feed on cell content, followed by whiteflies and aphids, which are phloem-feeding herbivores. Whether phloem sap constitution varies between plants exposed to different levels of UV-B needs investigation. Leaf-chewing insects were not abundant on the broccoli plants, but they might respond differently and be able to discriminate between plants of different UV-B pretreatments. Contrasting results have been found for the effects of plant UV-treatment on leaf chewers. Chewing herbivores are either deterred by high UV-exposed plants (Ballaré *et al.*, 1996; Caputo *et al.*, 2006; Foggo *et al.*, 2007) or not affected by UV treatment (Reifenrath and Müller, 2009).

The characteristic defence metabolites of Brassicaceae, glucosinolates, were mostly unaffected by UV-B. This has also been shown earlier for the combined effects of UV-A and UV-B on various species of Brassicaceae (Reifenrath and Müller, 2007; Kuhlmann and Müller, 2009). The low constitutive concentrations of glucosinolates in all greenhouse-grown broccoli plants may have been primarily responsible for the similar insect infestation patterns after outdoor exposure despite the different UV-B pretreatment. Most likely, insect infestation led to a strong induction of glucosinolates in all broccoli plants. Even though plants were already infested with insects after 24 h, the 3-fold induction of indolyl glucosinolates could only be detected at 72 h after field exposure and insect infestation (Table 2; Fig. 1). The total glucosinolate amounts per plant, mainly represented by indolyl glucosinolates, correlated with the number of attacking insect individuals per plant at the final harvest. Different abiotic factors, such as temperature or wind, may also have influenced the metabolite changes in field-exposed broccoli plants. A high induction of indolyl glucosinolates by herbivorous insects, i.e. leaf chewers, has already been described for several Brassicaceae (Textor and Gershenzon, 2009). Thrips were the most abundant herbivores on broccoli, therefore indolyl glucosinolate production was probably largely caused by these insects. However, the combination of multiple insect attacks may also have strong induction potential. Recently it has been shown that thrip feeding induces an increase in jasmonate levels in *A. thaliana* (Abe *et al.*, 2008). Jasmonate is an important signalling hormone, also leading to the induction of indolyl glucosinolates in various Brassicaceae (Textor and Gershenzon, 2009; van Dam *et al.*, 2009). However, the effects of thrips on glucosinolate concentrations have never been directly investigated.

Flavonoid amounts per plant were not correlated with insect infestation and flavonoid concentrations were not correlated with glucosinolate concentrations. Broccoli thus responded specifically to UV-B with an induction of flavonoids and to insect infestation and outdoor-exposure with an induction of glucosinolates, showing separate stimulus-specific responses. Earlier studies reported an overlap in gene-expression due to UV-B and herbivore feeding (Izaguirre *et al.*, 2003), but plants are obviously able to distinguish between impact by UV-B radiation and stress by herbivorous insects (Pandey and Baldwin, 2008). More studies are needed to disentangle the plant responses to different environmental impacts on a molecular and a metabolite level.

Increases of defence metabolites might also increase the plant quality for human nutrition. Flavonoids, as well as aliphatic and indolyl glucosinolates and their hydrolysis products of broccoli, potentially have important benefits for human health due to their anti-inflammatory and anti-tumorigenic properties (Gomes *et al.*, 2008; Jeffery and Araya, 2009). Growth of plants in innovative greenhouses transmitting higher UV-B radiation levels can increase plant quality with regard to flavonoid concentrations, whereas these UV-B conditions will not improve glucosinolate quantities. Higher UV-B radiation during early plant growth did not protect broccoli plants against the attack of various herbivorous insects when transferred to outdoor conditions. Longer field exposure of plants that received high UV-B at the early growth stage may, however, reveal other values for these plants.

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