

# Unlike Quercetin Glycosides, Cyanidin Glycoside in Red Leaf Lettuce Responds More Sensitively to Increasing Low Radiation Intensity before than after Head Formation Has Started

Christine Becker,<sup>\*,†</sup> Hans-Peter Klaering,<sup>†</sup> Monika Schreiner,<sup>†</sup> Lothar W. Kroh,<sup>§</sup> and Angelika Krumbein<sup>†</sup>

<sup>†</sup>Leibniz Institute for Vegetable and Ornamental Crops Grossbeeren/Erfurt e.V., Theodor-Echtermeyer-Weg 1, 14979 Grossbeeren, Germany

<sup>§</sup>Institute of Food Technology and Food Chemistry, Berlin University of Technology, Gustav-Meyer-Allee 25, 13355 Berlin, Germany

**ABSTRACT:** This study investigated the effect of low-level photosynthetic photon flux density (PPFD; 43–230  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) on the major phenolic compounds of red leaf lettuce in three growth stages, before, during, and after head formation, using HPLC-DAD-ESI-MS<sup>2</sup> and evaluating via multiple regression analysis. Generally, the light-related increase of flavonoid glycosides was structure and growth stage-dependent. In detail, an interaction was detected between plant age and PPFD regarding cyanidin-3-O-(6''-O-malonyl)-glucoside concentration: the increase was strongest before head formation. The relationship between PPFD and quercetin-3-O-(6''-O-malonyl)-glucoside concentration was linear, whereas the increase of quercetin-3-O-glucoside and -3-O-glucuronide concentrations abated with increasing PPFD. Independent of growth stage, the caffeic acid derivatives concentration was not related to PPFD. All major phenolic compounds decreased with plant age. These results show the differential regulation of cyanidin, quercetin, and caffeic acid derivatives in lettuce, although closely connected biosynthetically, and emphasize the importance of ontogeny in the study of plant physiology.

**KEYWORDS:** flavonoid glycoside, caffeic acid derivative, cyanidin, quercetin, red leaf lettuce, low radiation intensity, PPFD, multiple regression analysis, growth stage

## INTRODUCTION

In Central Europe, lettuce is cultivated in greenhouses in cool seasons. Unfortunately, conventional greenhouses consume a lot of energy: Besides labor, energy consumption is accountable for a substantial share of costs during crop production.<sup>1</sup> With increasing costs for fossil fuel, the public interest in CO<sub>2</sub> balances of food, and the political will to reduce CO<sub>2</sub> emissions, producers can face enormous socio-economic pressure, and the need for new approaches and ideas arises.<sup>1–3</sup>

The German ZINEG project is developing strategies to operate greenhouses in a more climate friendly manner ([www.zineg.de](http://www.zineg.de)). When greenhouses have to be heated in cool seasons, the application of transparent energy-saving screens constitutes a large energy-saving potential; unfortunately, they also reduce the photosynthetic radiation (PAR) available for crops.<sup>2</sup> Hence, photosynthesis of crops is reduced and less biomass accumulation results in lower yields.<sup>4</sup> Additionally, secondary metabolites, which are important for produce quality, may be influenced by radiation, for example, flavonoids and phenolic acids. They are synthesized via the phenylpropanoid pathway, in which phenylalanine ammonia-lyase is an important early enzyme having a light-dependent expression.<sup>5</sup>

Epidemiological studies strongly link a diet rich in polyphenols with a low incidence of coronary heart disease or cancer.<sup>6</sup> In vitro tests with single substances corroborate these beneficial effects.<sup>7</sup> Nevertheless, synergistic and additive effects of dietary polyphenols are considered to play a major role.<sup>6</sup> Hence, it appears wise to generally enhance the accumulation of phenolic substances in fruits and vegetables by horticultural approaches instead of administering single

substances as dietary supplement. To do this we need to understand how their biosynthesis responds to different cultivational strategies.

Major phenolic compounds in red leaf lettuce are glycosides of cyanidin, quercetin, and luteolin as well as caffeic acid derivatives.<sup>8</sup> The cyanidin glycosides are especially important for visual quality as they are responsible for the red appearance of the leaves. Previous studies reported an enhancing effect of radiation on flavonoid glycosides and caffeic acid derivatives in lettuce. However, they addressed photosynthetic photon flux densities (PPFD) as high as 800  $\mu\text{mol m}^{-2} \text{s}^{-1}$ <sup>9</sup> or focused on radiation from the ultraviolet (UV) range of the spectrum.<sup>10,11</sup> None of these approaches is relevant in the scenario we want to focus on: In cool seasons, PPFD in Central European greenhouses is rather low, especially when energy-saving screens are applied, and their glass cover has very low transmittance for UV radiation. PPFD reduction from 420 to 225  $\mu\text{mol m}^{-2} \text{s}^{-1}$  elicits a structure-dependent response of major phenolic compounds in red leaf lettuce.<sup>12</sup> However, it is not clear if further reductions would still have the same effects. The influence of low-level PPFD may be very different from that of high-level PPFD because, generally, phenolics are ascribed a photoprotective function.

**Special Issue:** International Workshop on Anthocyanins (IWA2013)

**Received:** October 24, 2013

**Revised:** December 31, 2013

**Accepted:** January 1, 2014

**Published:** January 1, 2014

During ontogeny, plants live through different phases with distinct morphological and physiological features.<sup>13</sup> Although crop plants are often harvested before they have transitioned to the generative phase or completed reproduction, their development can be divided into growth stages with pronounced differences regarding morphology and physiology. The concentration of flavonoids and phenolic acids, for instance, is subject to variation throughout plant development.<sup>14,15</sup> Because it is commonly consumed as a leaf vegetable, lettuce is harvested before it enters generative growth. Nonetheless, there are distinguishable growth stages related to head formation as described by Krug et al.:<sup>16</sup> The process of head formation is based on reorientation of leaves from their primary horizontal rosette state toward an upright position, progressively bending inward to form a head, which will grow and become larger and denser with an increasing number of leaves. Obviously, this affects at least the distribution of radiation within the plant. In red leaf lettuce, young leaves have a lower quercetin aglycone concentration than older ones,<sup>10</sup> and the overall concentration of flavonol glycosides and caffeic acid derivatives of whole heads is higher in lettuce plants in early growth stages than in later growth stages.<sup>12</sup>

Although the light dependency of flavonoid biosynthesis in general is well-known, Kubasek et al.<sup>17</sup> additionally found the developmental stage to influence the induction of pivotal flavonoid biosynthesis genes in *Arabidopsis thaliana* (Brassicaceae) seedlings. When it comes to studying development-dependent effects of radiation on plants, many studies have focused on UV radiation.<sup>10,17,18</sup> Interestingly, Reifenrath and Müller<sup>18</sup> detected an interaction between UV radiation and leaf age regarding their effect on flavonol concentration in *Nasturtium officinale* (Brassicaceae). This indicates that the same elicitor could lead to a different response from the same plant, depending on the plant's growth stage. Indeed, flavonoid aglycones in red leaf lettuce display a development-dependent response to UV radiation.<sup>10</sup>

In the presented experiment, we studied the relationship between the concentration of several flavonoid glycosides as well as caffeic acid derivatives and low-level PPFD in three growth stages of two red leaf lettuce cultivars in a climate-controlled greenhouse. We wanted to examine the following hypotheses: (I) The concentrations of the major phenolic compounds of red leaf lettuce respond to PPFD levels below  $225 \mu\text{mol m}^{-2} \text{s}^{-1}$ , as are common in Central European greenhouses in cool seasons. (II) Flavonoid glycosides and caffeic acid derivatives respond in a structure-dependent manner. (III) Their PPFD response is development-dependent.

## MATERIALS AND METHODS

**Plant Cultivation.** We conducted the experiment in Grossbeeren (52°20' N, 13°18' E), Germany, in March–April 2012. The mean temperature in the greenhouse was 16.3 °C (minimum, 11.7 °C; maximum, 23.6 °C). Red oak leaf and red lollo lettuce (*Lactuca sativa* L. var. *crispa* L. cv. Eventai RZ and *L. sativa* L. var. *crispa* L. cv. Satine, respectively; RijkZwaan, De Lier, The Netherlands) was sown in rockwool cubes, kept at ca. 10 °C for 2 days for germination, and subsequently grown in a conventional greenhouse until the experiment started. When plants had developed four true leaves and a mean aboveground mass of 0.9 g 5 weeks after sowing, they were transferred into the experimental setting, where they were grown hydroponically, using nutrient film technique. The nutrient solution was prepared according to the method of Sonneveld and Straver<sup>19</sup> and checked for macronutrient concentration every week. The greenhouse area was divided into four blocks, according to differences in radiation intensity

due to greenhouse construction elements. Additionally, half of each block was covered with a shading net, reducing the photosynthetic photon flux (PPFD) by 45%. This way, we obtained data on flavonoid glycoside and caffeic acid derivative concentrations in two cultivars of red leaf lettuce related to eight PPFD levels, ranging from 43 to  $230 \mu\text{mol m}^{-2} \text{s}^{-1}$ . We followed the natural light cycle; no additional lighting was supplied. The PPFD was monitored permanently in each block with light meters LI 190SA Quantum (LI-COR Inc., Lincoln, NE, USA) and recorded by a data logger (DATATAKER, Victoria, Australia). Greenhouse glass has a very good transmissibility for visible and near-infrared wavebands but allows only 64% of UV-A and <1% of UV-B and UV-C radiation, respectively, to pass. Of the UV wavebands, UV-B is generally considered to be most bioactive.<sup>10</sup> We harvested the aboveground organs at three dates, obtaining data on different growth stages within the horticulturally interesting vegetative growth phase: Harvest 1 took place 12 days after planting (DAP), before head formation started. Harvest 2 took place shortly after head formation started (21 DAP), and at harvest 3, mature heads were formed (35 DAP). Harvest dates were set on the basis of experience gained in previous experiments.

**Plant Growth Characteristics.** At all harvest dates, the mean head weight of three plants per PPFD level and cultivar was used to obtain the mean head mass ( $n = 8$ ). Values for head mass are given in grams of fresh matter (FM). To obtain dry matter (DM) content, weight before and after lyophilization was compared. Values for DM content are given as percent of FM.

Additionally, we counted the number of leaves (minimum length = 1.5 cm) and measured the maximum diameter of three lettuce heads for each PPFD level and cultivar ( $n = 8$ ).

Light use efficiency was calculated for plants before and after the onset of head formation. This value displays how much dry matter plants accumulated per intercepted mole of PPFD. For each block and cultivar the intercepted PPFD was calculated separately, based on the measured head diameter. The PPFD absorbing surface area ( $\text{m}^2$ ) was approximated by a circle. This area was multiplied with the PPFD integral ( $\text{mol m}^{-2}$ ) for the intervals between the first and second and the second and third harvest dates, respectively. Light use efficiency ( $\text{g mol}^{-1}$ ) was obtained as the ratio of the plants' dry matter gain (g) between the respective harvest dates and the corresponding intercepted PPFD.

**Sample Preparation.** A mixed sample from three plants was prepared for each cultivar, treatment, and replication. Only limp or deteriorated outer leaves were removed. Within 30 min after harvesting, the plants were cut in smaller pieces, mixed, frozen at  $-20$  °C until lyophilized (Christ Beta 1-16, Osterode, Germany), and ground with an ultracentrifuge mill (hole size = 0.25 mm; ZM 200, Retsch, Haan, Germany).

**Analyses of Phenolic Compounds.** Flavonol and flavone glycosides as well as caffeic acid derivatives were analyzed via HPLC-DAD-ESI-MS according to the method of Becker, Kläring, Kroh, and Krumbein.<sup>12</sup> In short, 0.5 g of lyophilized, pulverized lettuce powder was extracted with 25 mL of aqueous methanol (50% MeOH) for 90 min at room temperature. The extract was centrifuged, and the supernatant was cleaned with PTFE filters and analyzed via HPLC-DAD-ESI-MS<sup>2</sup>. The anthocyanin method was similar, except for a slightly different composition of the extraction agent and a shorter extraction time: The extraction agent was acidified aqueous methanol (40% MeOH, 10% acetic acid). Extraction of anthocyanin glycosides took 15 min.

The system used for analysis consists of an Agilent HPLC series 1100 Ion Trap (Agilent, Waldbronn, Germany). The compounds were separated on a Prodigy column (ODS 3,  $150 \times 3$  mm,  $5 \mu\text{m}$ , 100 Å; Phenomenex, Aschaffenburg, Germany) with a security guard C18 (ODS 3,  $4 \times 3$  mm,  $5 \mu\text{m}$ , 100 Å) at 30 °C using a water/acetonitrile gradient. DAD wavelength for quantification was 330 nm for caffeic acid derivatives, 350 nm for flavonol and flavone glycosides, and 520 nm for anthocyanidin glycosides.

**Statistical Analyses.** To study the effect of plant ontogeny, radiation intensity, cultivar, and their interactions on flavonoid glycosides and caffeic acid derivatives, multiple regression analysis

was performed. Representing plant growth stage, DAP was included to study the influence of ontogeny. Radiation intensity is included as PPFD (given in  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). On the basis of previous experience<sup>12</sup> we considered the mean PPFD during the last 12 days before harvest to be an important factor influencing flavonoid biosynthesis. The two cultivars entered the analysis as cultivar 1 (red oak leaf) and cultivar 2 (lollo rosso), respectively. The resulting equations presented in this study contain only terms having an influence on the investigated variable that significantly differs from zero. A significance level of  $\alpha = 0.01$  was applied.

Growth characteristics were evaluated via two-way ANOVA (Fisher's *F* test).

Calculations were performed using STATISTICA (version 10, Statsoft Inc., Tulsa, OK, USA).

## RESULTS AND DISCUSSION

**Influence of Cultivar.** Throughout ontogeny, red oak leaf lettuce had a higher number of leaves and greater diameter and head mass than lollo rosso (Table 1). The concentration of

**Table 1. Plant Growth Characteristics of Two Red Leaf Lettuce Cultivars, Grown in Low Mean Daytime PPFD ( $43\text{--}230 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) for 12, 21, and 35 Days after Planting (DAP), Respectively: Number of Leaves, Diameter, and Head Mass in Fresh Matter (FM) (Mean  $\pm$  SD of the Eight PPFD Levels,  $n = 8$ )**

DAP	cultivar	no. of leaves	diameter (cm)	head mass (g FM)
12	red oak leaf	$10.3 \pm 0.2$	$28.8 \pm 2.8$	$9.2 \pm 1.5$
21	red oak leaf	$18.6 \pm 2.5$	$37.7 \pm 3.7$	$52.6 \pm 24.3$
35	red oak leaf	$30.7 \pm 5.0$	$41.3 \pm 2.1$	$199.7 \pm 66.5$
12	lollo rosso	$7.3 \pm 0.5$	$16.6 \pm 1.2$	$7.5 \pm 2.1$
21	lollo rosso	$11.5 \pm 2.1$	$26.7 \pm 0.3$	$33.8 \pm 13.1$
35	lollo rosso	$21.0 \pm 5.1$	$32.9 \pm 0.6$	$137.0 \pm 65.5$

phenolic compounds in lettuce can be greatly influenced by genotype,<sup>20</sup> but lollo rosso and red oak leaf lettuce showed only quantitative and no qualitative differences. Concentrations were mostly higher in red lollo rosso, which is largely in line with results by DuPont et al.<sup>21</sup> Multiple regression analysis listed the cultivar as an influential factor on quercetin-3-*O*-(6''-*O*-malonyl)-glucoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-glucuronide/luteolin-7-*O*-glucuronide, and chicoric acid concentrations (Table 2).

**Plant Growth Characteristics.** Table 1 presents the head mass, number of leaves, and head diameter per cultivar at the three harvest dates. As intended, the plants were in three distinct growth stages. Twelve days after planting, head mass and number of leaves of both cultivars were still low. The leaves formed an open rosette in which all leaves were exposed to radiation. From 12 to 21 DAP, head mass increased only moderately while there was a remarkable increase in diameter. The rosette was much denser at the second harvest date with leaves partly overlapping. From 21 to 35 DAP, there was a marked increase in head mass but not so much in diameter. Independent of cultivar, there was a significantly higher increase in head mass from 21 to 35 DAP compared to the earlier period, whereas the increase in diameter was higher from 12 to 21 DAP compared to the later period.

These results corroborate our visual assessment that plants had not yet developed a head 12 DAP, but that this process had begun at 21 DAP and continued until 35 DAP and is in agreement with the literature.<sup>16</sup> In the following, we named the three studied growth stages accordingly: preheading (harvest 12

**Table 2. Results of Multiple Regression Analysis: Equations Present the Significantly Influential Factors on the Single Phenolic Compounds of Red Leaf Lettuce ( $n = 48$ ,  $\alpha = 0.01$ )<sup>a</sup>**

compound	equation	R <sup>2</sup>
Cy3MG	$= 1.075774 - 0.148608 \times \text{DAP} + 0.019459 \times \text{PPFD} - 0.000028 \times \text{PPFD}^2 + 0.003040 \times \text{DAP}^2 - 0.000077 \times \text{PPFD} \times \text{DAP}$	0.88
Q3G	$= 0.061983 - 0.012242 \times \text{DAP} + 0.033398 \times \text{cultivar} + 0.001004 \times \text{PPFD} - 0.000002 \times \text{PPFD}^2 + 0.000215 \times \text{DAP}^2$	0.69
Q3MG	$= 6.472664 - 0.765445 \times \text{DAP} + 2.181619 \times \text{cultivar} + 0.020639 \times \text{PPFD} + 0.013099 \times \text{DAP}^2$	0.77
Q3Gc/L7Gc	$= 0.761703 - 0.107507 \times \text{DAP} + 0.236988 \times \text{cultivar} + 0.007146 \times \text{PPFD} - 0.000015 \times \text{PPFD}^2 + 0.001859 \times \text{DAP}^2$	0.82
chicoric acid	$= 1.003199 - 0.108660 \times \text{DAP} + 0.893134 \times \text{cultivar} + 0.002311 \times \text{DAP}^2 - 0.021650 \times \text{cultivar} \times \text{DAP}$	0.75
chlorogenic acid	$= 0.932527 - 0.019782 \times \text{DAP}$	0.45
caffeoylmalic acid	$= 0.170214 - 0.001731 \times \text{DAP}$	0.17

<sup>a</sup>DAP, days after planting; PPFD, photosynthetic photon flux density in  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ; cultivar, red oak leaf (1) and lollo rosso (2); Cy3MG, cyanidin-3-*O*-(6''-*O*-malonyl)-glucoside; Q3G, quercetin-3-*O*-glucoside; Q3MG, quercetin-3-*O*-(6''-*O*-malonyl)-glucoside; Q3Gc/L7Gc, quercetin-3-*O*-glucuronide/luteolin-7-*O*-glucuronide. The value of R<sup>2</sup> displays the coefficient of determination.

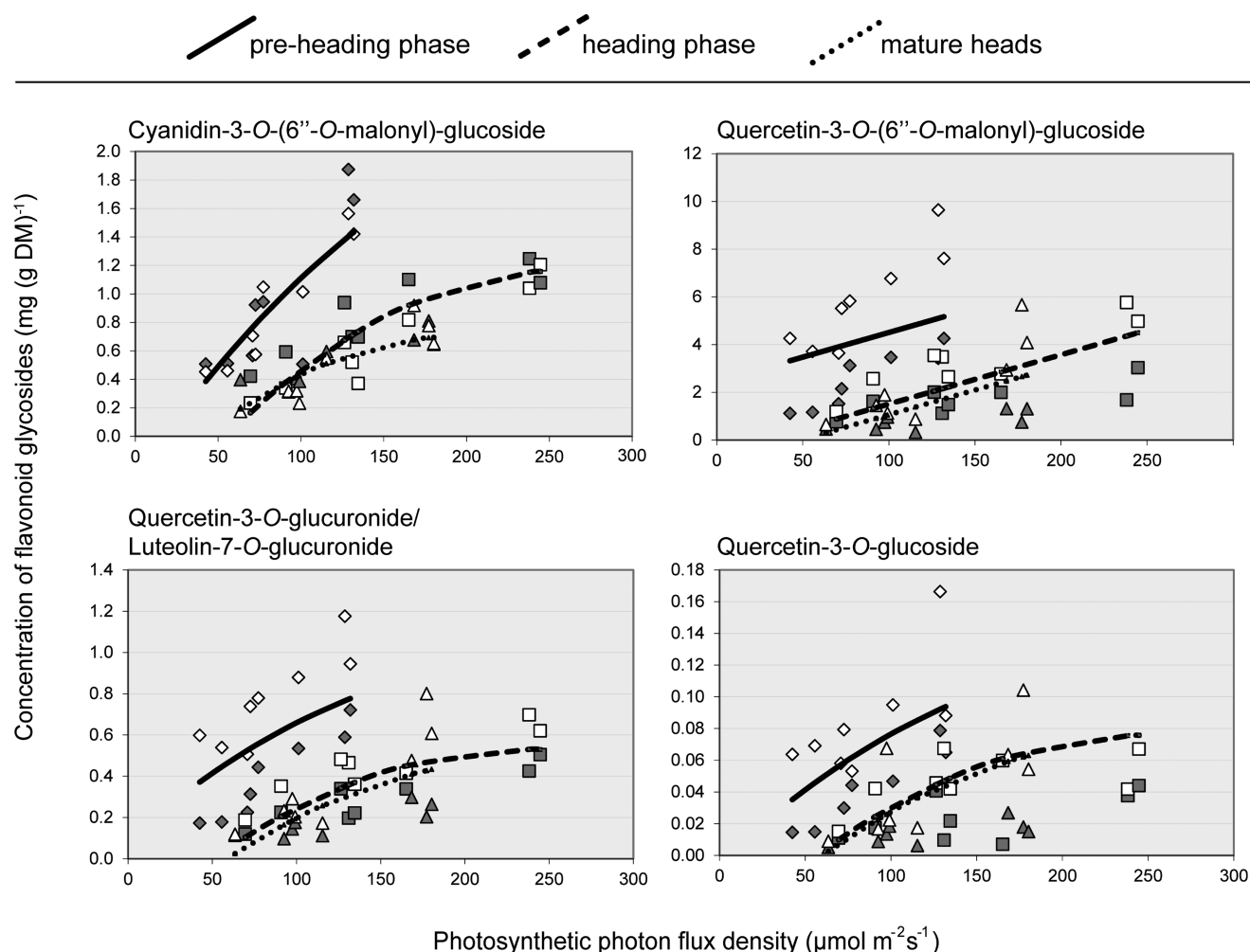
DAP) and heading stages (harvest 21 DAP) and mature heads (harvest 35 DAP), respectively.

The light use efficiency between the first and second harvests was significantly lower than that between the second and third harvest dates ( $0.3$  and  $0.7 \text{ g mol}^{-1}$ , respectively). No significant differences between the cultivars were detected. This indicates that the photosynthetic capacity in the first interval was lower than in the second, as plants accumulated less biomass per intercepted mole of PPFD.

**Phenolic Compounds.** In our HPLC-DAD-ESI-MS<sup>3</sup> analyses of flavonol, flavone, and anthocyanidin glycosides as well as caffeic acid derivatives in red leaf lettuce, we identified three quercetin glycosides, one luteolin glycoside, one cyanidin glycoside, and several caffeic acid derivatives. The main phenolic compound was chicoric acid (di-*O*-caffeoyltartaric acid), followed by quercetin-3-*O*-(6''-*O*-malonyl)-glucoside, cyanidin-3-*O*-(6''-*O*-malonyl)-glucoside, quercetin-3-*O*-glucuronide, luteolin-7-*O*-glucuronide, chlorogenic acid (5-*O*-caffeoylquinic acid), caffeoylmalic acid, and quercetin-3-*O*-glucoside. These compounds were previously reported for red leaf lettuce.<sup>8,21</sup> Quercetin-3-*O*-glucuronide and luteolin-7-*O*-glucuronide coeluted and were quantified as sum. Mass spectrometric data suggested they on average contributed equal shares to the peak evaluated via DAD, which is in line with data obtained by DuPont, Mondin, Williamson, and Price.<sup>21</sup>

**Flavonoid Glycosides.** The results for flavonoid glycosides are presented in Figure 1 and Table 2. Generally, all flavonoid glycosides were present in higher concentrations in plants in earlier growth stages than in later ones, with the larger difference between preheading stage and heading stage than between heading stage and mature heads. Remarkably, the concentrations of all flavonoid glycosides increased with PPFD even in the very low levels studied. The response depended on structure and growth stage.





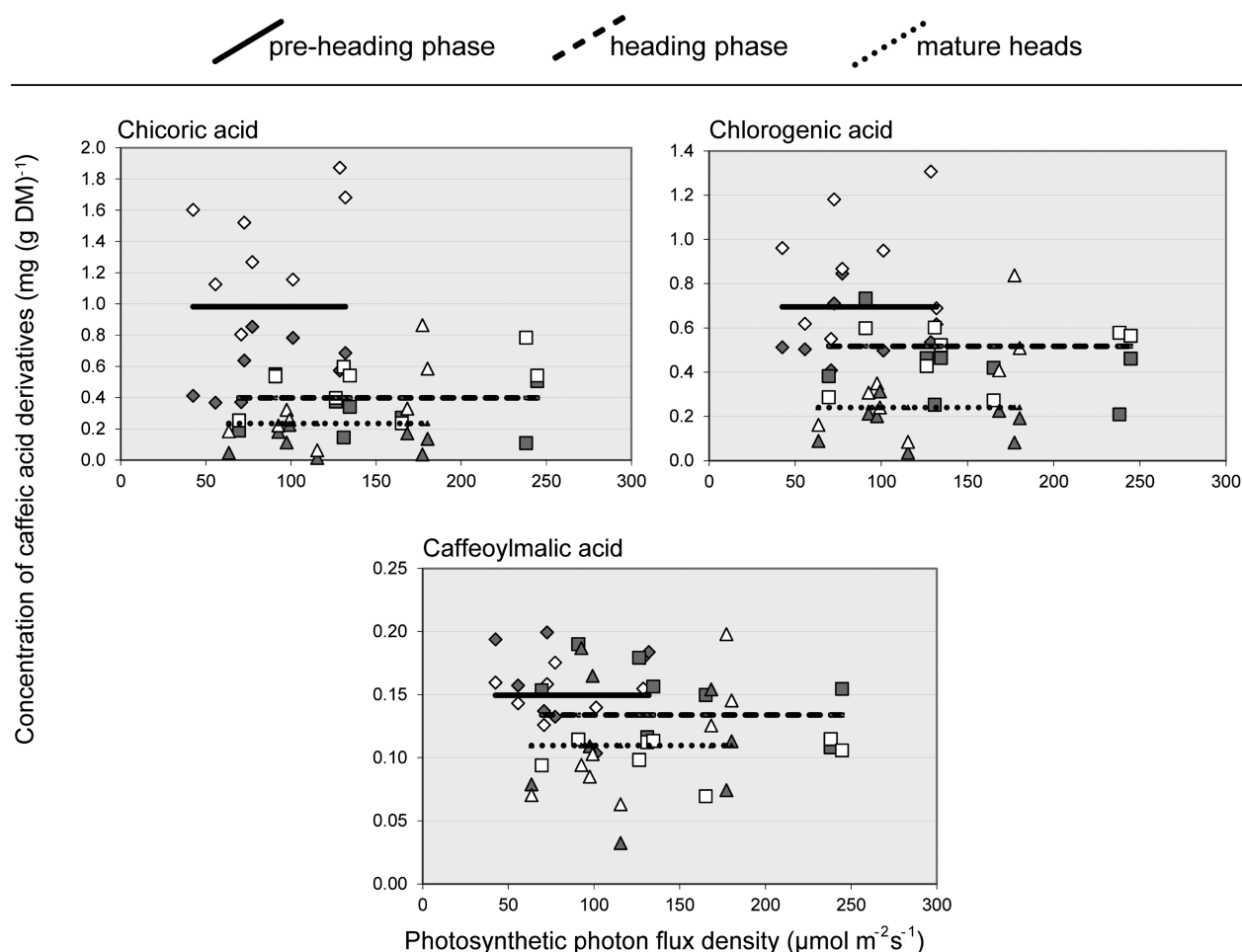
**Figure 1.** Concentration of flavonoid glycosides per gram dry matter (DM) of red leaf lettuce in three different growth stages in relation to photosynthetic photon flux density (in  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). The relationship was evaluated via multiple regression analysis. Gray symbols represent values of red oak leaf lettuce; white symbols represent lollo rosso values. Diamonds represent values of preheading phase, squares represent values of heading phase, and triangles represent values of mature heads. The solid line is based on the values of preheading stage, the broken line is based on values of heading stage, and the dotted line is based on values of mature heads.

In detail, with regard to cyanidin-3-*O*-(6''-*O*-malonyl)-glucoside, the lettuce plants' response to radiation varied between the growth stages considered. Multiple regression analysis revealed an interaction between PPFD and plant age: Increasing PPFD had a stronger influence on smaller plants than larger ones. Furthermore, the increase of cyanidin-3-*O*-(6''-*O*-malonyl)-glucoside concentration with rising PPFD was becoming slower with rising PPFD (due to the negative influence of the square term of PPFD), suggesting the approach of a saturation level in PPFD ranges higher than those studied here. Due to the positive influence of the square term of DAP, the differences of cyanidin-3-*O*-(6''-*O*-malonyl)-glucoside concentration between growth stages were more pronounced between preheading and heading phases than between heading and mature heads.

The interaction between PPFD and plant age is most interesting and has not been reported this clearly before. With regard to UV radiation, Behn, Schurr, Ulbrich, and Noga<sup>10</sup> detected a similar age-dependent response when comparing leaves of different age. They cultivated red leaf lettuce for 21 days under UV-B exclusion and subsequently transferred the plants to an environment with ambient UV-B intensity for 2 days. Following this short-term exposure to UV-B radiation, the

cyanidin concentration increased only in inner (= young) leaves of lettuce heads. Because responsiveness generally decreased with increasing leaf and plant age, they concluded that younger leaves have an increased requirement for cyanidin as photoprotectant as they have a lower capacity to utilize radiation energy compared to older ones. The light use efficiency of young lettuce was indeed much lower than that of older plants: Per intercepted mole of PPFD, older plants accumulated more than double the dry matter younger ones did. This increase during ontogeny is in line with the literature.<sup>22</sup> Concordantly, the PPFD required for maximum photosynthesis of lettuce plants increases with plant age.<sup>23</sup> This implies an imbalance between captured light energy and its utilization in carbon fixation in early growth stages as has been suggested for juvenile tree leaves by Hughes et al.<sup>24</sup> The surplus energy poses an oxidative threat to plant cells. This suggests that young plants were protecting themselves by anthocyanin accumulation and may explain the higher responsiveness of young plants, even to such low radiation intensities as we studied.

Although the role of anthocyanins in planta is to some degree still subject to discussion, their protective role against photoinhibition and photooxidation in leaves is widely accepted.<sup>25</sup> They absorb light in the green and yellow



**Figure 2.** Concentration of caffeic acid derivatives per gram dry matter (DM) of red leaf lettuce in three different growth stages in relation to photosynthetic photon flux density (in  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). The relationship was evaluated via multiple regression analysis. Gray symbols represent values of red oak leaf lettuce; white symbols represent lollo rosso values. Diamonds represent values of preheading phase, squares represent values of heading phase, and triangles represent values of mature heads. The solid line is based on the values of preheading stage, the broken line is based on values of heading stage, and the dotted line is based on values of mature heads.

wavebands (500–600 nm) and can, therefore, shield the photosynthetic apparatus against surplus radiation, which cannot be used for carbon assimilation and may therefore lead to oxidative damage of photosynthetic tissue.<sup>26</sup> On the other hand, they have a high antioxidant capacity<sup>27</sup> and can counteract reactive oxygen species formed, for example, by photochemistry.<sup>28</sup> Especially during light-sensitive ontogenetic stages, for example, in juvenile leaves in which the photosynthetic apparatus is not yet fully functional, these qualities become obvious.<sup>24</sup> Young leaves are more valuable to plants than old leaves and should, hence, be very effectively protected against harmful impacts.<sup>18</sup>

The concentrations of quercetin-3-*O*-(6''-*O*-malonyl)-glucoside, quercetin-3-*O*-glucoside, and quercetin-3-*O*-glucuronide/luteolin-7-*O*-glucuronide also increased with rising PPFD and decreased with plant age. Between preheading and heading plants the difference regarding concentration was very pronounced. Between heading plants and mature heads, on the other hand, there is hardly any detectable difference. The general increase of the concentration of quercetin glycosides with radiation is in line with previous results.<sup>9,10</sup> Yet, it has not been demonstrated before with regard to such low PPFD levels. Other than for cyanidin glycosides, we did not detect an interaction between PPFD and plant age with regard to

quercetin glycoside concentration. Accordingly, Behn, Schurr, Ulbrich, and Noga<sup>10</sup> also found the UV-B response of cyanidin to be more strongly influenced by leaf age than the response of quercetin: Quercetin concentration increased in all of the studied leaf ages.

The effects we detected were clearly structure dependent. On the one hand, they were related to the aglycone: Cyanidin glycosides responded plant age-dependently, whereas quercetin glycosides did not. On the other hand, the glycosidic moiety appears to play a role, too. Otherwise, the three quercetin glycosides would have shown the same response. Yet, in the radiation levels we studied, quercetin-3-*O*-(6''-*O*-malonyl)-glucoside concentration increased linearly with PPFD, whereas the quercetin-3-*O*-glucoside and quercetin-3-*O*-glucuronide/luteolin-7-*O*-glucuronide concentrations were negatively influenced by the square term of PPFD. Hence, the increase of their concentration was not linear but abated with rising PPFD. This implies that the latter compounds approached a saturation level at higher PPFD than we studied.

All of the presented results were related to dry matter. Yet, for consumers, the concentration-related fresh matter is also interesting. In our experiment dry matter content was not influenced by PPFD but decreased with increasing plant age (data not shown). Therefore, considering the concentration per

100 g of fresh matter, the influence of PPFD corresponds to the results obtained for data related to dry matter, whereas the influence of plant age was even more pronounced.

All studied flavonoid glycosides displayed a remarkable, unexpectedly strong response to changes in PPFD level between approximately 50 and 150  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , which was independent of plant age. This demonstrates an impressive PAR sensitivity of the regulative mechanisms.

As mentioned earlier (Introduction and Influence of Cultivar), one major aspect of progressing plant age in the vegetative growth phase of lettuce is the increasing number of leaves accompanied by a change in plant architecture: the formation of a more or less dense head. As a result, the distribution of radiation within the plant is changed. With more leaves shading each other, less biomass per head is directly exposed to radiation. The light-dependent flavonoid biosynthesis, however, will only take place in the exposed tissue.<sup>29</sup> The relative decrease of light-exposed, flavonoid-synthesizing tissue per plant may explain the decrease of flavonoid glycosides we measured with progressing head formation.

Yet, according to Kubasek, Ausubel, and Shirley,<sup>17</sup> flavonoid biosynthesis regulation is influenced by environmental as well as developmental factors. When studying the mRNA level of four flavonoid biosynthesis genes in *Arabidopsis thaliana* seedlings, they found that mRNA levels were higher in 3-day-old than in 7-day-old seedlings, although plants were exposed to identical radiation conditions. They concluded that the gene expression potential was related to plant age. Despite obvious differences between the plants studied by Kubasek, Ausubel, and Shirley<sup>17</sup> and our experiment (different radiation conditions and different plant species), these results may at least be partly transferrable to lettuce in more advanced growth stages: The interaction between plant age and PPFD regarding cyanidin glycoside allows for the speculation that the decreasing concentration with plant age may not only be due to the mentioned dilution effect but also be influenced by growth-stage-dependent variation in the induction capacity of genes involved in biosynthesis. As we detected an interaction only between plant age and radiation concerning cyanidin glycosides, we suppose that the decrease of quercetin glycosides with plant age is due to the relative decrease of radiation-exposed tissue in more developed heads, whereas the decrease in cyanidin glycoside concentration is (additionally) mediated by decreasing biosynthetic capacity with increasing leaf age.

In further studies, it would therefore be interesting to take a closer look and compare the induction of genes at the junction leading to quercetin or cyanidin biosynthesis, for instance, the mRNA levels of flavonol synthase and dihydroflavonol 4-reductase, respectively.<sup>15</sup> Yet, considering the high number of levels at which regulatory processes can take place following transcription (stability of mRNA, translator effectivity, enzyme activation, and more), it might be additionally interesting to study the quantity and activity of the respective enzymes.

**Caffeic Acid Derivatives.** The results for caffeic acid derivatives are presented in Figure 2 and Table 2. Independent of growth stage, PPFD had no significant influence on the concentration of any of the caffeic acid derivatives. Unlike PPFD, progressing ontogeny had an influence on the concentrations of all three caffeic acid derivatives: They decreased with plant age, however to various degrees.

The decrease of chicoric acid concentration was counteracted by the positive influence of the square term of DAP. Hence, it was markedly higher in the preheading than in the heading

stage, whereas the latter did not differ as much from the mature heads' concentration. We observed a significant influence of the cultivar on chicoric acid concentration. Chlorogenic and caffeoylmalic acid were only significantly influenced by DAP, that is, plant age.

The result that none of the three studied caffeic acid derivatives was influenced by PPFD is not in line with Oh, Carey, and Rajashekar,<sup>9</sup> who found an increase of caffeic acid derivatives with increased radiation. However, they exposed their small lettuce plants to 800  $\mu\text{mol m}^{-2} \text{s}^{-1}$  for 1 day; this is much higher than the PPFD incident in our experiment (50–250  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). According to Fu et al.,<sup>30</sup> lettuce suffers no stress under 100–400  $\mu\text{mol m}^{-2} \text{s}^{-1}$  but serious stress under 800  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Furthermore, greenhouse glass absorbs UV radiation to a large degree and phenolic acids are discussed as UV protectants in red leaf lettuce.<sup>31</sup> Hence, incident radiation in this experiment may have lacked the intensity and UV wavelengths needed to trigger caffeic acid derivative biosynthesis.

In conclusion, we could partly confirm our first hypothesis: Quercetin and cyanidin glycosides responded to PPFD at low levels, whereas caffeic acid derivatives did not. Consequently, the measured responses were structure-dependent and the second hypothesis could be fully confirmed. The third hypothesis that the PPFD response is development dependent could be confirmed only for cyanidin-3-*O*-(6"-*O*-malonyl)-glucoside, which responded differently to increasing levels of PPFD depending on the plant's growth stage. Our results highlight how important it is to consider the plant's developmental stage when its physiology is studied.

For lettuce cultivation in greenhouses and the application of energy-saving screens, this implies that screen application during red leaf lettuce production is possible without reducing the concentration of chicoric, chlorogenic, or caffeoylmalic acid, no matter in which growth stage plants are shaded by the screen. Unfortunately, this is not true for cyanidin and quercetin glycosides. Yet, in mature heads losses due to reduced radiation were smaller than with younger plants with regard to three of four flavonoid glycosides.

The high concentration of polyphenols in preheading leaf lettuce and their response to shading may be especially interesting for producers of baby leaf lettuce. Furthermore, the selection of cultivars that are less sensitive to shading and maintain higher concentrations of phytonutrients under these conditions may represent interesting prospects for future studies.

## AUTHOR INFORMATION

### Corresponding Author

\*(C.B.) E-mail: becker@igzev.de. Phone: +49 (0) 33701 78362.

### Funding

This research was supported by the German Federal Ministry for Environment, Nature Conservation and Nuclear Safety and the Rentenbank managed by the Federal Ministry of Food, Agriculture and Consumer Protection with the assistance of the Federal Agency for Agriculture and Food.

### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

We thank Kersten Maikath, Uwe Kunert, Ingrid Rathenow, Angela Schmidt, Anna Hahn, Juliane Hepting, Susanne Neugart, and Elke Büsch very much for their valuable help and support.

## ABBREVIATIONS USED

Q3G, quercetin-3-O-glucoside; Q3MG, quercetin-3-O-(6''-O-malonyl)-glucoside; Q3Gc, quercetin-3-O-glucuronide; L7Gc, luteolin-3-O-glucuronide; Cy3MG, cyanidin-3-O-(6''-O-malonyl)-glucoside; PPFD, photosynthetic photon flux density; PAR, photosynthetically active radiation; UV, ultraviolet (radiation); mRNA, messenger ribonucleic acid; SD, standard deviation; DAP, days after planting; FM, fresh matter; DM, dry matter; ACN, acetonitrile; MeOH, methanol; HPLC-DAD-ESI-MS, high-performance liquid chromatograph with diode array detector coupled via electrospray ionization interface to mass spectrometer

## REFERENCES

- (1) Meyer, J. Extremely insulated greenhouse concept with non-fossil fuel heating system. *Acta Hortic.* **2011**, 201–208.
- (2) Bakker, J. C.; Adams, S. R.; Boulard, T.; Montero, J. L. Innovative technologies for an efficient use of energy. *Acta Hortic.* **2007**, 801, 49–62.
- (3) Frangi, P.; Piatti, R.; Amoroso, G. Evaluation of different screens for energy saving in the greenhouse. *Acta Hortic.* **2011**, 275–279.
- (4) Klaering, H.-P.; Krumbein, A. The effect of constraining the intensity of solar radiation on the photosynthesis, growth, yield and product quality of tomato. *J. Agron. Crop Sci.* **2013**, DOI: 10.1111/jac.12018.
- (5) Leyva, A.; Jarillo, J. A.; Salinas, J.; Martinezzapater, J. M. Low temperature induces the accumulation of phenylalanine ammonia lyase and chalcone synthase messenger RNAs of *Arabidopsis thaliana* in a light dependant manner. *Plant Physiol.* **1995**, 108, 39–46.
- (6) Boudet, A. M. Evolution and current status of research in phenolic compounds. *Phytochemistry* **2007**, 68, 2722–2735.
- (7) Mulabagal, V.; Ngouajio, M.; Nair, A.; Zhang, Y.; Gottumukkala, A. L.; Nair, M. G. *In vitro* evaluation of red and green lettuce (*Lactuca sativa*) for functional food properties. *Food Chem.* **2010**, 118, 300–306.
- (8) Llorach, R.; Martínez-Sánchez, A.; Tomás-Barberán, F. A.; Gil, M. I.; Ferreres, F. Characterisation of polyphenols and antioxidant properties of five lettuce varieties and escarole. *Food Chem.* **2008**, 108, 1028–1038.
- (9) Oh, M.-M.; Carey, E. E.; Rajashekar, C. B. Environmental stresses induce health-promoting phytochemicals in lettuce. *Plant Physiol. Biochem.* **2009**, 47, 578–583.
- (10) Behn, H.; Schurr, U.; Ulbrich, A.; Noga, G. Development-dependent UV-B responses in red oak leaf lettuce (*Lactuca sativa* L.): physiological mechanisms and significance for hardening. *Eur. J. Hortic. Sci.* **2011**, 76, 33–40.
- (11) Tsormpatsidis, E.; Henbest, R. G. C.; Battey, N. H.; Hadley, P. The influence of ultraviolet radiation on growth, photosynthesis and phenolic levels of green and red lettuce: potential for exploiting effects of ultraviolet radiation in a production system. *Ann. Appl. Biol.* **2010**, 156, 357–366.
- (12) Becker, C.; Kläring, H.-P.; Kroh, L. W.; Krumbein, A. Temporary reduction of radiation does not permanently reduce flavonoid glycosides and phenolic acids in red lettuce. *Plant Physiol. Biochem.* **2013**, 72, 154–160.
- (13) Hackett, W. P. Differential expression and functional significance of anthocyanins in relation to phasic development in *Hedera helix* L. *Adv. Bot. Res.* **2002**, 37, 95–102.
- (14) Krumbein, A.; Saeger-Fink, H.; Schonhof, I. Changes in quercetin and kaempferol concentrations during broccoli head ontogeny in the three broccoli cultivars. *J. Appl. Bot. Food Qual.* **2007**, 81, 136–139.
- (15) Treutter, D. Managing phenol contents in crop plants by phytochemical farming and breeding – visions and constraints. *Int. J. Mol. Sci.* **2010**, 11, 807–857.
- (16) Krug, H.; Liebig, H.-P.; Stützel, H. *Gemüseproduktion*; Eugen Ulmer GmbH: Stuttgart, Germany, 2002.
- (17) Kubasek, W. L.; Ausubel, F. M.; Shirley, B. W. A light-independent developmental mechanism potentiates flavonoid gene expression in *Arabidopsis* seedlings. *Plant Mol. Biol.* **1998**, 37, 217–223.
- (18) Reifenrath, K.; Müller, C. Species-specific and leaf-age dependent effects of ultraviolet radiation on two Brassicaceae. *Phytochemistry* **2007**, 68, 875–885.
- (19) Sonneveld, C.; Straver, N. *Nutrient Solutions for Vegetables and Flowers Grown in Water or Substrates*; Glasshouse Crops Research Station: Naaldwijk, The Netherlands, 1988; Vol. 7, p 16.
- (20) Gazula, A.; Kleinhenz, M. D.; Streeter, J. G.; Miller, A. R. Temperature and cultivar effects on anthocyanin and chlorophyll b concentrations in three related Lollo Rosso lettuce. *Hortscience* **2005**, 40, 1731–1733.
- (21) DuPont, S. M.; Mondin, Z.; Williamson, G.; Price, K. R. Effect of variety, processing and storage on the flavonoid glycoside content and composition of lettuce and endive. *J. Agric. Food Chem.* **2000**, 48, 3957–3964.
- (22) Wheeler, T. R.; Hadley, P.; Ellis, R. H.; Morison, J. I. L. Changes in growth and radiation use by lettuce crops in relation to temperature and ontogeny. *Agric. For. Meteorol.* **1993**, 66, 173–186.
- (23) Sanchez, C. A.; Allen, R. J.; Schaffer, B. Growth and yield of crisphead lettuce under various shade conditions. *J. Am. Soc. Hortic. Sci.* **1989**, 114, 884–890.
- (24) Hughes, N. M.; Morley, C. B.; Smith, W. K. Coordination of anthocyanin decline and photosynthetic maturation in juvenile leaves of three deciduous tree species. *New Phytol.* **2007**, 175, 675–685.
- (25) Hatier, J.-H. B.; Gould, K. S. Anthocyanin function in vegetative organs. In *Anthocyanins*; Gould, K., Davies, K., Winefield, C., Eds.; Springer Science + Business Media: New York, 2009.
- (26) Gould, K. S. Nature's Swiss army knife: the diverse protective roles of anthocyanins in leaves. *J. Biomed. Biotechnol.* **2004**, 5, 314–320.
- (27) Rice-Evans, C.; Miller, N.; Paganga, G. Antioxidant properties of phenolic compounds. *Trends Plant Sci.* **1997**, 2, 152–159.
- (28) Neill, S. O.; Gould, K. S. Anthocyanins in leaves: light attenuators or antioxidants? *Funct. Plant Biol.* **2003**, 30, 865–873.
- (29) Hohl, U.; Neubert, B.; Pforte, H.; Schonhof, I.; Böhm, H. Flavonoid concentrations in the inner leaves of head lettuce genotypes. *Eur. Food Res. Technol.* **2001**, 213, 205–211.
- (30) Fu, W.; Li, P.; Wu, Y. Effects of different light intensities on chlorophyll fluorescence characteristics and yield in lettuce. *Sci. Hortic.* **2012**, 135, 45–51.
- (31) García-Macías, P.; Ordidge, M.; Vysini, E.; Waroophan, S.; Battey, N. H.; Gordon, M. H.; Hadley, P.; John, P.; Lovegrove, J. A.; Wagstaffe, A. Changes in the flavonoid and phenolic acid contents and antioxidant activity of red leaf lettuce (Lollo Rosso) due to cultivation under plastic films varying in ultraviolet transparency. *J. Agric. Food Chem.* **2007**, 55, 10168–10172.