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CO_2 enrichment can produce high red leaf lettuce yield while increasing most flavonoid glycoside and some caffeic acid derivative concentrations

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ABSTRACT

Carbon dioxide (CO_2) enrichment is a common practice in greenhouses to increase crop yields up to 30%. Yet, reports on the effect on foliar phenolic compounds vary. We studied the effect on two red leaf lettuce cultivars, grown for 25 days in growth chambers at CO_2 concentrations of 200 or 1000 ppm, with some plants exchanged between treatments after 11 days. As expected, head mass increased with higher CO_2 concentration. Regression analysis, corrected for head mass, showed increased concentrations of most flavonoid glycosides at high CO_2 concentrations while only some caffeic acid derivatives were increased, and not uniformly in both cultivars. Sugar concentrations increased with CO_2 concentration. Generally, conditions in the 10 days before harvest determined concentrations. We suspect that phenolic compounds were mainly accumulated because plenty of precursors were available. The results indicate that CO_2 enrichment can result in high yields of red leaf lettuce rich in phenolic compounds.

1. Introduction

Carbon dioxide (CO_2) enrichment is a commonly used method to increase yields of greenhouse cultivated crops (Chalabi, Biro, Bailey, Aikman, & Cockshull, 2002), featuring CO_2 concentrations of up to 1000 ppm in the greenhouse air. Increases of up to 30% are well possible in northern countries during autumn, winter and spring in fruit vegetables (Willits & Peet, 1989) and also in lettuce (Hunt, Wilson, Hand, & Sweeney, 1984). Recently, closed greenhouses were developed. During high solar radiation periods, those greenhouses capture and store thermal energy to reuse it for heating during dark and cold periods (Schmidt et al., 2011). The closed operation mode allows for maintaining high CO_2 concentrations year round resulting in very high yields (De Gelder, Dieleman, Bot, & Marcelis, 2012). However, without CO_2 supply to a greenhouse with closed ventilation, the CO_2 concentration in the air can decrease down to 150 ppm during the day due to the CO_2 uptake by plants, as shown for cucumber (Kläring, Hauschild, Heißner, & Bar-Yosef, 2007).

In contrast to the effect of the CO_2 concentration on photosynthesis and yield, there are fewer reports on its effect on secondary metabolites in vegetables. In general, the effect of CO_2 concentration on secondary metabolites seems to be low compared to other environmental factors. Thus, Krumbein, Schwarz, and Kläring (2006) could not find any effect on carotenoid content in tomato. In broccoli, rising CO_2 concentration increased the total glucosinolate concentration which however, was counteracted by a decrease of concentration of indole glucosinolates (Schonhof, Kläring, Krumbein, & Schreiner, 2007). The existing reports on the effect of CO_2 enrichment on the concentration of foliar flavonoids and phenolic acids also show mixed results, ranging from increases to decreases or no effect.

In grapevine (*Vitis vinifera* L.), increased concentrations of flavonoids were detected (Bindi, Fibbi, & Miglietta, 2001). In strawberries (*Fragaria x ananassa*), Wang, Bunce, and Maas (2003) found increased anthocyanin and flavonol concentrations and Kuokkanen, Julkunen-Tiitto, Keinänen, Niemelä, and Tahvanainen (2001) measured increased flavonol glycoside concentrations in birch seedlings (*Betula pendula* Roth).

Peñuelas et al. (1996) tested the responses of several species and found differences: Elevated CO_2 concentrations resulted in increased concentrations of total phenolics in wheat leaves (*Triticum aestivum*), decreased concentrations in pine tree needles (*Pinus eldarica* L.) and had no effect regarding orange tree leaves (*Citrus aurantium* L.). In high CO_2 concentrations, Peltonen, Vapaavuori, and Julkunen-Tiitto (2005) observed increased concentrations of phenolic acids, flavonols, flavanols and condensed

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tannins but decreased concentrations of flavones in birch leaves while Sallas, Luomala, Utriainen, Kainulainen, and Holopainen (2003) detected decreased total phenolics concentration in Norway Spruce (*Picea abies*) but not in Scots Pine (*Pinus sylvestris* L.). The species-related heterogeneity may be increased by different time spans the plants were exposed to elevated CO_2 concentrations, different developmental stages tested in different environments, i.e. growth chambers, greenhouses, open fields, pots or field-soil, as well as unspecific measurements, i.e. total phenolics or total flavonoids instead of single compounds, of different plant organs (Goufo et al., 2014). On average, however, elevated atmospheric CO_2 concentration may stimulate the production of flavonoids and other phenolics (DeLucia, Nabity, Zavala, & Berenbaum, 2012).

Epidemiological studies strongly link a diet rich in phenolic compounds with a low incidence of coronary heart disease or cancer (Boudet, 2007). Although *in vitro* tests with single substances corroborate these beneficial effects (Mulabagal et al., 2010), they have to be considered carefully as *in vivo* studies do not always reproduce these effects (Rimbach, Melchin, Moehring, & Wagner, 2009). According to *in vitro* tests, synergistic and additive effects of dietary phenolic compounds are considered to play a major role (Boudet, 2007) but high doses of flavonols may be disadvantageous due to possible pro-oxidative effects (Rietjens et al., 2005). Hence, it appears wise to generally enhance their accumulation in fruits and vegetables by horticultural approaches instead of administering single substances as dietary supplement. To achieve this we need to monitor how their concentrations change in response to different cultivation strategies. Major phenolic compounds in red leaf lettuce are glycosides of cyanidin, quercetin, and luteolin as well as esters of caffeic acid (Llorach, Martínez-Sánchez, Tomás-Barberán, Gil, & Ferreres, 2008). The cyanidin glycosides are especially important for visual quality as they are responsible for the red color of the leaves (Gould & Lister, 2006).

CO_2 enrichment leads to increased photosynthesis rates (Nederhoff & Vegter, 1994). Hence, there is an abundance of assimilates which can be funneled into biosynthetic pathways of all sorts (Treutter, 2010). This becomes obvious by enhanced biomass accumulation and may result in higher concentrations of secondary metabolites due to higher availability of precursor molecules. The pathway for the biosynthesis of flavonoids and caffeic acid derivatives, the phenylpropanoid pathway, is reported to be directly linked to the carbohydrate status of plants: Monosaccharides are in several steps transformed into phenylalanine via the shikimate pathway and the first step of the phenylpropanoid pathway is de-amination of phenylalanine to obtain cinnamic acid (Schopfer & Brennicke, 2010). Additionally, sugar has been found to directly upregulate transcription factors involved in anthocyanin biosynthesis in *Arabidopsis thaliana* (Solfanelli, Poggi, Loreti, Alpi, & Perata, 2006).

According to the International Panel on Climate Change (IPCC), the global atmospheric CO_2 concentration is rising (IPCC, 2007). However, horticultural crops are under-represented when it comes to studying the effects of elevated atmospheric CO_2 concentration on phenolic compounds which leaves the IPCC with little data to predict future crop yields and quality (Goufo et al., 2014). As far as we know, there are no studies regarding the effect of CO_2 concentration on phenolic compounds in lettuce to date.

In a study on the effect of irradiance on phenolic compounds in lettuce, we found that their concentration depends rather on the radiation intensity during the last days before harvest than on the average of the total cultivation period (Becker, Klaering, Kroh, & Krumbein, 2013).

In the presented study, we tested the following hypotheses under controlled conditions: (I) the CO_2 concentration has a positive effect on the concentration of flavonoid glycosides and caffeic acid derivatives in lettuce, (II) CO_2 enrichment during lettuce cultivation is more efficient in the weeks before harvest than in the weeks after planting, and (III) high CO_2 concentrations increase the concentrations of sugars which serve as precursors for the phenylpropanoid pathway. We used two cultivars because different genotypes may respond differently (Jaafar, Ibrahim, & Karimi, 2012). Instead of measuring total concentrations, we measured single phenolic compounds using HPLC-DAD-ESI-MS^3. Based on our own and the experience of others (Becker et al., 2013; Caporn, 1989) we cultivated lettuce for several weeks at a light intensity that was saturating but not stressful, at low and high CO_2 concentration, respectively, and standard cultivation temperature, to obtain results of practical relevance.

2. Material and methods

2.1. Plant cultivation

Red Oak Leaf and red Lollo lettuce seeds (*Lactuca sativa* L. var. *crispa* L. cv. Eventai RZ and *L. sativa* L. var. *crispa* L., cv. Satine, respectively; RijkZwaan, De Lier, The Netherlands) were sown in rockwool cubes, kept at 10 °C for two days for germination and subsequently grown in a conventional greenhouse until the experiment started. When plants had developed five true leaves (five weeks old) and weighed about 1.6 g they were transferred into four growth chambers (Weiss Gallenkamp, Loughborough, UK) where they were grown in 2 L containers in aerated nutrient solution. The nutrient solution was prepared according to Sonneveld and Straver (1988). It contained the following ions in mmol L^{-1} 19.0 NO_3^-, 1.25 NH_4^+, 2.0 $H_2PO_4^-$. 1.125 SO_4^{2-}, 11.0 K^+, 4.5 Ca^{2+}, 1.0 Mg^{2+}, and in µmol L^{-1} 40.0 Fe^{3+}, 1.0 Mn^{2+}, 4.0 Zn^{2+}, 30.0 $B(OH)_4^-$, 0.75 Cu^{2+}, and 0.5 MoO_4^{2-}, resulting in an electrical conductivity of the nutrient solution of 2.3 dS m^{-1}. The pH of the nutrient solution was 5.5. Solution taken up by the plants was replenished periodically. Air temperature in the chambers was kept at 20 °C during daytime and 15 °C at night, relative humidity was approximately 80% and radiation was supplied by halogen metal vapor lamps Osram Powerstar HQI-BT 400 W/D Pro (Osram, Munich, Germany) and krypton lamps. The light cycle consisted of four elements: 11 h of darkness, 0.5 h of dawn, 12 h of light and another 0.5 h of twilight. During the light phase, the mean photosynthetic photon flux density (PPFD) was 260 µmol m^{-2} s^{-1}. During dusk and dawn only the krypton lamps were switched on, resulting in a mean PPFD of 95 µmol m^{-2} s^{-1}. These radiation intensities were measured with a portable light meter LI-250 (LI–COR Inc., Lincoln, Nebraska, USA). The daily light integral was 11.6 mol m^{-2}.

Technical pure CO_2 was supplied to all chambers during the light phase to compensate for the uptake by the plants. The set points for the control of the CO_2 concentration were 1000 ppm and 200 ppm each in two chambers. In the latter, the CO_2 concentration during the dark phase increased to about 500 ppm due to the plant's respiration and the lack of a CO_2 absorber. Shortly after the beginning of the light phase it arrived at 200 ppm as result of the plants' photosynthesis and was then kept at this level by the CO_2 controller.

Each chamber held 12 plants of each cultivar. Per cultivar, four plants were harvested 11 days after planting (DAP). At the same day four plants were exchanged between the CO_2 treatments. After 14 more days, all remaining plants were harvested. For each cultivar, 8 replicates consisting of 4 plants from two independently controlled chambers were installed. However, regarding Oak Leaf, treatment 1000_200 and 200_1000, only 6 and 7 plants, respectively, could be considered because the others were infected with a fungus. As the plants were cultivated in independent pots, the infection did not spread.

2.2. Plant growth characteristics

For all samples, above ground (lettuce heads) and below ground (roots) organs were harvested separately. At both harvest dates, eight plants per cultivar and treatment were weighed to obtain the mean head and root mass. Values are given in gram fresh matter (FM). Lettuce heads were cut in pieces. Some head fresh matter and the roots were dried in an oven at 80 °C for three days, to obtain dry matter content. Values for dry matter content are given in milligram dry matter per gram fresh matter.

2.3. Sample preparation

Within 30 min after harvesting, the remaining cut lettuce heads were frozen at −20 °C until lyophilized (Christ Beta 1–16, Osterode, Germany). Only limp or deteriorated outer leaves were removed during preparation. Dried plant matter was ground with an ultracentrifuge mill (hole size: 0.25 mm; ZM 200, Retsch, Haan, Germany).

2.4. Analyses of phenolic compounds

The well-established HPLC-DAD-ESI-MS^3 method for the determination of flavonol glycosides and phenolic acids, reported by Scattino et al. (2014) was optimized for lettuce. Best results were obtained by extracting 20 mg of lyophilized, pulverized lettuce powder three times (600, 300, and 300 µl) with aqueous methanol (50% MeOH; Carl Roth GmbH, Karlsruhe, Germany) for 40, 20, and 10 min in a thermomixer (1400 rpm, 20 °C; Eppendorf thermomixer comfort, Germany). Between the three extraction phases, they were centrifuged at 4.500 rpm and 20 °C for 10 min (Labofuge 400R, Heraeus Instruments, Thermo Fisher Scientific, Waltham, USA) and the supernatants were combined. To remove larger particles, the extracts were filtered through Spin-X centrifuge filters (0.22 µm; Geyer GmbH, Berlin, Germany) by centrifugation at 3.000 rpm and 20 °C for 5 min. Afterwards they were transferred to glass vials and analyzed via HPLC-DAD-ESI-MS^3.

The anthocyanin extracts were prepared similarly to the method applied to flavonols and phenolic acids, except for a slightly different composition of the extraction agent and a shorter extraction time: The extraction agent was acidified aquaeous methanol (40% MeOH, 10% acetic acid) to a pH value of 2.6. Extraction of anthocyanin glycosides took 3 × 10 min.

The system used for analysis consists of an Agilent HPLC series 1100 (Agilent, Waldbronn, Germany), containing of a degaser, binary pump, autosampler, thermostat and a photodiode array detector (DAD). The components were separated on a Ascentis Express F5 column with a C18 security guard (150 × 4.6 mm, 5 µm; 4 × 4.6 mm, 5 µm; Supelco Analytical, Sigma Aldrich, Munich, Germany) at 30 °C using a water/acetonitrile gradient. Solvent A consisted of 99.5% water and 0.5% acetic acid (Merck, Darmstadt, Germany) whereas solvent B was 100% acetonitrile (ACN; J.T. Baker, Deventer, The Netherlands). Two separate gradients were used for flavonol glycosides and phenolic acids (gradient 1) and anthocyanins (gradient 2), respectively. Gradient 1 held the following percentages of ACN: 5% (2 min), 5–12% (13 min), 12–20% (31 min), 20–90% (3.5 min), 90% isocratic (2.5 min), 90–5% (0.7 min), isocratic 5% (6.3 min). Gradient 2 was distinctly shorter: 5–20% B (2 min), 20% B isocratic (1 min), 20–90% B (3.5 min), 90% B isocratic (2.5 min), 90–5% B (1 min) and 5% isocratic (3 min). Flow rate in both gradients was 0.85 ml/ min. Flavonol glycosides and phenolic acids were detected in the mass spectrometer as deprotonated molecular ions and characteristic mass fragment ions using an Agilent series 1100 MSD (ion trap) with ESI as ion source in negative mode. Nitrogen served as dry gas (10 l/min; 350 °C) and nebulizer gas (40 psi). Helium was used as collision gas in the ion trap. Mass optimization was performed for quercetin 3-*O*-glucoside $[M-H]^-$ *m/z*. Anthocyanidin glycosides were identified using the positive mode. Identification of the compounds was achieved by comparing retention time, absorption maxima and mass spectra to that of standard substances, when available, or to literature data (DuPont, Mondin, Williamson, & Price, 2000; Llorach et al., 2008). Standard substances were purchased at Carl Roth GmbH (Karlsruhe, Germany; quercetin-3-*O*-glucoside) and Sigma–Aldrich GmbH (Munich, Germany; di-*O*-caffeoyltartaric acid and cyanidin-3-*O*-glucoside).

The DAD was used for quantification, using the detection wavelengths 330 nm (phenolic acids), 350 nm (flavonol and flavone glycosides) and 520 nm (anthocyanidin glycosides). External calibration curves were prepared in the respective relevant concentrations, using the standard substances where available. Cyanidin-3-*O*-(6″-*O*-malonyl)-glucoside was quantified as cyanidin-3-*O*-glucoside. Quercetin-3-*O*-(6″-*O*-malonyl)-glucoside, quercetin-3-*O*-glucuronide and luteolin-7-*O*-glucuronide were quantified quercetin-3-*O*-glucoside equivalents. The caffeic acid derivatives were quantified as di-*O*-caffeoyltartaric acid equivalents.

2.5. Analysis of sugars

For sugar analysis, 10 mg of freeze-dried plant material were extracted with 800 µl of aqueous ethanol (80% EtOH; Carl Roth GmbH, Karlsruhe, Germany), vortexed and incubated for 20 min at 78 °C. After centrifugation at 14000 rpm and 4 °C for 10 min, the supernatant was transferred into a new Eppendorf reaction tube. The extraction was repeated twice with 400 µl 50% EtOH. Of the combined supernatants, 5 µl were and analyzed via enzymatic essay in microplates as described by Klopotek and Kläring (2014).

2.6. Statistical analyses

In order to detect significant differences regarding growth characteristics due to the different CO_2 concentrations, one-way ANOVA was performed (Fisher's *F*-test) for each cultivar and harvest date separately, followed by Tukey's Honest Significant Difference test with a significance level of $\alpha = 0.05$. In order to detect significant differences regarding phenolic compounds and sugars due to the different CO_2 concentrations, regression analysis was performed for each cultivar separately. In order to test hypothesis (II) that conditions shortly before harvest have much greater impact on phenolic compound concentrations than those after planting, all data were related to the CO_2 concentration during the last 10 days before plant sampling at 11 and 25 DAP. In addition, polyphenol concentrations are strongly influenced by plant development (Becker, Klaering, Schreiner, Kroh, & Krumbein, 2014b). Therefore we included the plants' head mass (logarithmic) into this analysis. Regression coefficients were evaluated using Student's *t*-test. A significance level of $\alpha = 0.05$ was applied. Single plants were considered biological replicates. Calculations were performed using STATISTICA (version 10, Statsoft Inc., Tulsa, USA).

3. Results and discussion

3.1. Growth characteristics

As expected, we observed higher head mass (FM) with plants cultivated at 1000 ppm compared to 200 ppm CO_2 concentration, in both cultivars and at both harvest dates (Fig. 1). Compared to the 200 ppm treatment, plants from the 1000 ppm treatment on average gained a 72% higher head mass. Hunt et al. (1984) reported

a 30% higher absolute crop growth rate for lettuce under elevated CO_2 concentration. We observed a larger gain in head mass. This is partly due to the fact that our low CO_2 concentration of 200 ppm lies way below the commonly used "ambient" CO_2 concentration of about 335 ppm in these experiments. The main explanation, however, is probably that the CO_2 concentration of 1000 ppm in early growth stages resulted in increased leaf area due to increased photosynthesis. This increased leaf area in turn potentisized the effect of the elevated CO_2 concentrations in later growth stages. As an approximation, at 11 DAP the mean head diameters were on average over both cultivars 18.3 and 19.0 cm at 200 and 1000 ppm CO_2 concentration, respectively. At 25 DAP, head diameters were significantly different between plants cultivated at CO_2 concentrations of 200 ppm all the time or only the 14 days prior to harvest (27.1 and 26.2 cm) and plants cultivated at 1000 ppm all the time or only the 14 days prior to harvest (29.7 and 29.1 cm; data not shown). Head mass in red Lollo of both exchange treatments (1000_200 ppm and 200_1000 ppm) did not differ from each other and lay between those of plants permanently cultivated at 200 ppm and at 1000 ppm CO_2 concentration (Fig. 1). Regarding Oak Leaf, head mass of plants from the 1000_200 ppm and 200_1000 ppm treatments, respectively, did not differ from plants cultivated all the time at 200 and 1000 ppm CO_2 concentration, respectively (Fig. 1).

Shoot dry matter content of neither Oak Leaf nor Lollo was significantly influenced by CO_2 concentration, neither at 11 nor at 25 DAP. Mean shoot dry matter at 11 DAP was 66 mg g^{-1} (Oak Leaf) and 62 mg g^{-1} (Lollo) while it was 56 mg g^{-1} (Oak Leaf) and 50 mg g^{-1} (Lollo) at 25 DAP (data not shown).

The shoot/root-ratio on a dry matter basis of neither Oak Leaf nor Lollo was significantly influenced by CO_2 concentration, neither at 11 nor at 25 DAP. Mean shoot/root-ratio at 11 DAP was 7.7 g g^{-1} (Oak Leaf) and 7.9 g g^{-1} (Lollo) while it was 9.9 g g^{-1} (Oak Leaf) and 11.7 g g^{-1} (Lollo) at 25 DAP (data not shown). Because of this, root fresh and dry matter content are not shown here.

3.2. Phenolic compounds

In our HPLC-DAD-ESI-MS^3 analyses of phenolic compounds in red leaf lettuce, we identified two 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 chlorogenic acid (5-*O*-caffeoylquinic acid), quercetin-3-*O*-(6″-*O*-malonyl)-glucoside, caffeoylmalic acid, cyanidin-3-*O*-(6″-*O*-malonyl)-glucoside, caftaric acid (caffeoyltartaric acid), quercetin-3-*O*-glucuronide, isochlorogenic acid (di-*O*-caffeoylquinic acid) and luteolin-7-*O*-glucuronide. These compounds were previously reported for red leaf lettuce (Llorach et al., 2008).

3.2.1. Flavonoid glycosides

Results of the regression analysis are depicted in Fig. 2. Equations of the regression curves and their coefficients of determination (R^2) as well as the *p*-values of the two involved factors are given in Table 1. Two regression curves are depicted in each graph: one for 200 and one for 1000 ppm. In both Oak Leaf and Lollo lettuce, cyanidin-3-*O*-(6″-*O*-malonyl)-glucoside was higher in plants grown at 1000 compared to 200 ppm CO_2 concentration and decreased with increasing physiological plant age (Table 1). The same is true for both quercetin glycosides in both cultivars. Luteolin-7-*O*-glucuronide concentration likewise decreased with increasing physiological plant age in both cultivars. However, it was only positively influenced by CO_2 concentration in Oak Leaf lettuce which also had higher concentrations than Lollo. Our results on glycosides of the flavonol quercetin and the anthocyanidin cyanidin are in line with results reported on birch leaves and strawberries (Kuokkanen et al., 2001; Peltonen et al., 2005; Wang et al., 2003). While flavones decreased in birch leaves with increasing CO_2 concentration (Peltonen et al., 2005), in our experiment the luteolin glycoside was only significantly affected by CO_2 concentration in one of two cultivars.

The concentration of flavonoid glycosides was mostly influenced by the CO_2 concentration in the 10 days prior to harvest. This is demonstrated by the position of the open symbols (Fig. 2). This is underlined by the fact that regression analyses with the mean CO_2 concentration over the growing period resulted in lower coefficients of determination (data not shown). This pattern corresponds with results from a previous experiment where flavonoid glycoside concentrations were determined by light intensity in the 10 days directly before harvest rather than by previous conditions (Becker et al., 2013).

Decreasing flavonoid concentrations with increasing physiological plant age (Fig. 2) have been observed in lettuce previously (Becker et al., 2014b).

Our results show a general enhancement of flavonoid biosynthesis in plants grown at high CO_2 concentrations which is in line with DeLucia et al. (2012). This effect appears to be especially strong regarding the cyanidin glycoside (Fig. 2). The results indicate that this is due to the aglycone rather than the glycosidic

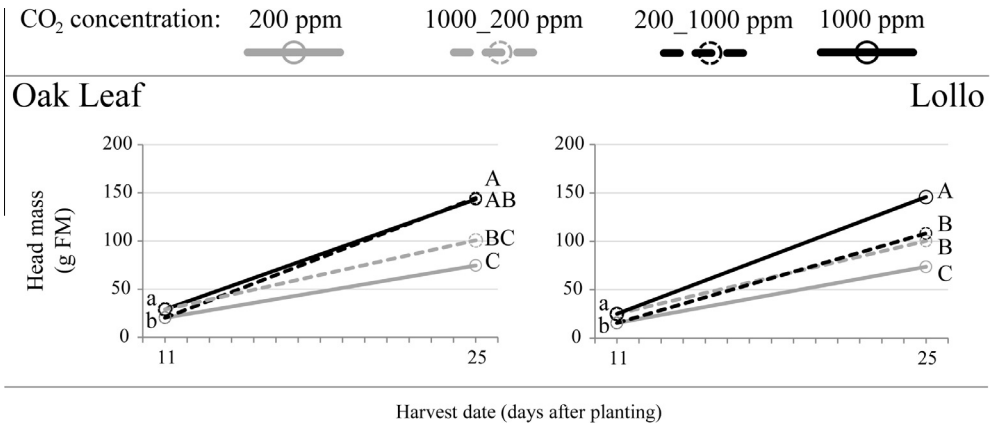

Fig. 1. Effect of CO_2 concentration (ppm) on mean head mass in gram fresh matter (g FM) of two red leaf lettuce cultivars (Oak Leaf and Lollo). The grey line represents plants cultivated at CO_2 concentrations of 200 ppm, the black line represents plants cultivated at 1000 ppm all the time. Broken lines represent plants that have been exchanged between treatments and thus been cultivated first at 200 then at 1000 ppm (broken black line) or first at 1000 then at 200 ppm CO_2 concentration (broken grey line). Identical letters indicate that no significant differences were detected between these treatments. Both harvest dates were evaluated separately (Tukey-test, $\alpha = 0.05$).

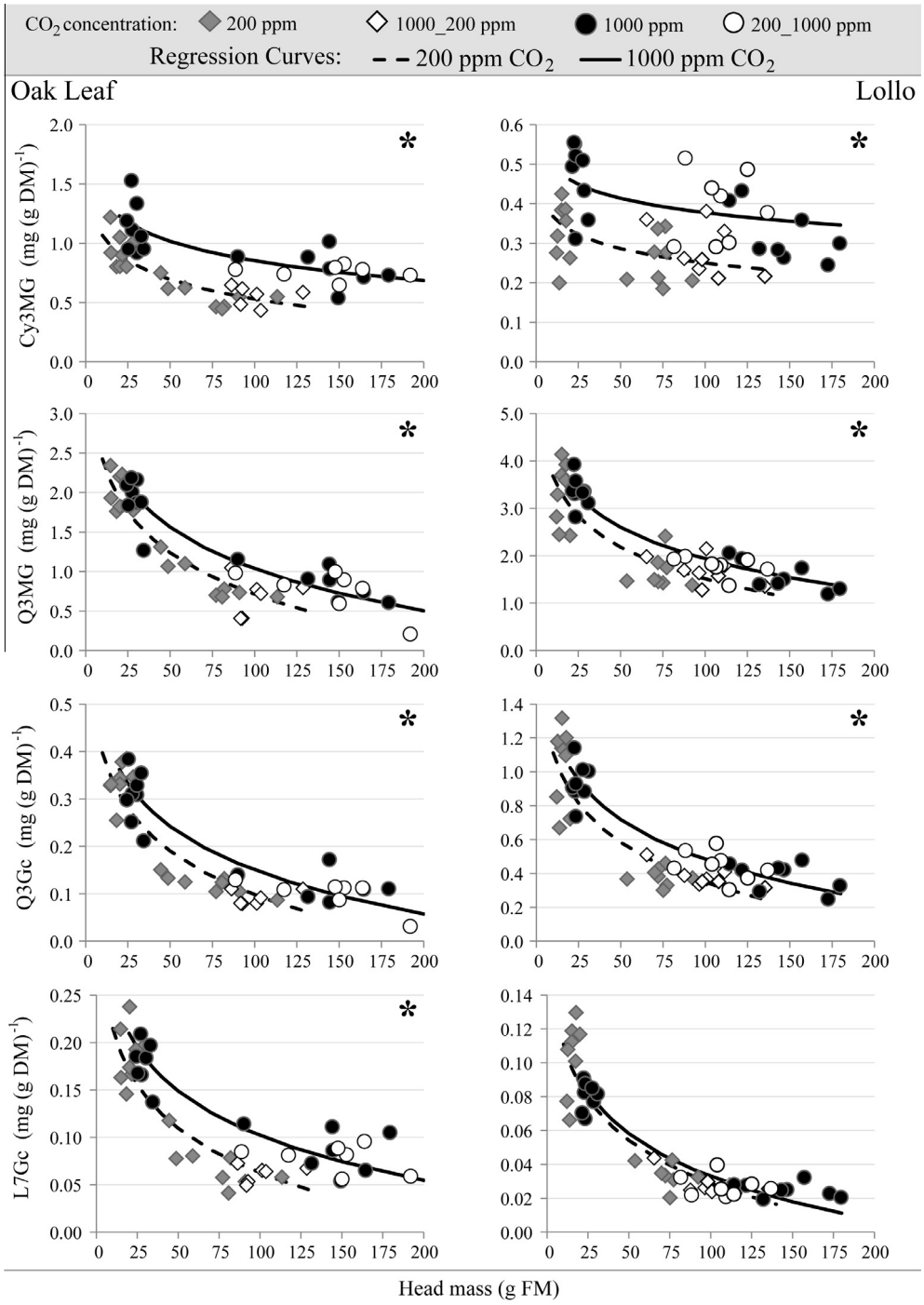

Fig. 2. Effect of CO_2 concentration (ppm) and head mass (g fresh matter) on the concentration of flavonoid glycosides per gram dry matter (g DM) in two red leaf lettuce cultivars (Oak Leaf and Lollo). Grey symbols represent plants cultivated at CO_2 concentrations of 200 ppm, black symbols represent plants cultivated at 1000 ppm all the time. White symbols represent plants that have been exchanged between treatments and thus been cultivated first at 1000 then at 200 ppm (diamonds) or first at 200 then at 1000 ppm (circles). The effect of CO_2 concentration and head mass on flavonoid glycoside concentrations was evaluated via multiple regression analysis (equations in Table 1). The solid and broken lines depict the flavonoid glycoside concentrations calculated with the regression equation for 1000 and 200 ppm CO_2 concentration, respectively. Asterisks mark significant differences between the CO_2 treatments Cy3MG: cyanidin-3-*O*-(6″-*O*-malonyl)-glucoside, Q3MG: quercetin-3-*O*-(6″-O-malonyl)-glucoside, Q3Gc: quercetin-3-*O*-glucuronide, L7Gc: luteolin-7-*O*-glucuronide.

sugar moiety: The two quercetin glycosides (glucuronide and malonylglucoside) responded similarly to each other while the response of the quercetin and cyanidin malonylglucosides differed.

Anthocyanin concentrations are high when radiation intensity is high, temperature is low or nutrients are deficient (Becker, Klaering, Kroh, & Krumbein, 2014a; Becker et al., 2014b; Peng et al., 2008). Neither condition applies to our experiment. Accumulation of flavonoids often coincides with conditions that enhance the formation of reactive oxygen species in plants (Agati et al., 2013). However, high CO_2 concentration is said to decrease the oxidative load in plants through unknown mechanisms (Farfan-Vignolo & Asard, 2012). The photoprotection theory suggests anthocyanins to be accumulated by plants to protect photosynthetic tissue by absorbing photosynthetically active radiation and/or scavenging reactive oxygen species or other radicals and it often serves as an explanation for anthocyanin accumulation

Table 1
Effects of CO_2 concentration during the 10 days before harvest (CO_2, ppm) and head mass at harvest (M_{head}, g) on the concentration of flavonoid glycosides and caffeic acid derivatives (mg (g DM)$^{-1}$). Coefficients were estimated using quasilinear regression analysis separately for each cultivar based on all samples harvested 11 and 25 DAP. Cy3MG: cyanidin-3-*O*-(6″-*O*-malonyl)-glucoside, Q3MG: quercetin-3-*O*-(6″-*O*-malonyl)-glucoside, Q3Gc: quercetin-3-*O*-glucuronide, L7Gc: luteolin-7-*O*-glucuronide. R^2 denotes the coefficient of determination.

Cultivar	Compound	Equation of regression curve	R^2	*p*-Value for	
				CO_2	M_{head}
Oak Leaf	Cy3MG	$=1.56 + 0.000402 * CO_2 - 0.242 * \ln(M_{head} + 1)$	0.72	<0.00001	<0.00001
	Q3MG	$=4.19 + 0.000405 * CO_2 - 0.772 * \ln(M_{head} + 1)$	0.88	0.00003	<0.00001
	Q3Gc	$=0.71 + 0.000065 * CO_2 - 0.135 * \ln(M_{head} + 1)$	0.85	0.0005	<0.00001
	L7Gc	$=0.37 + 0.000049 * CO_2 - 0.069 * \ln(M_{head} + 1)$	0.82	0.00001	<0.00001
	Chicoric acid	$=22.34 + 0.00266 * CO_2 - 3.942 * \ln(M_{head} + 1)$	0.77	0.0001	<0.00001
	Chlorogenic acid	$=4.46 + 0.000625 * CO_2 - 0.596 * \ln(M_{head} + 1)$	0.32	0.018	0.00004
	Caffeoylmalic acid	$=0.38 + 0.000027 * CO_2 + 0.082 * \ln(M_{head} + 1)$	0.06	0.745	0.060
	Caftaric acid	$=0.62 + 0.000016 * CO_2 - 0.068 * \ln(M_{head} + 1)$	0.50	0.422	0.00000
	Isochlorogenic acid	$=1.36 + 0.000199 * CO_2 - 0.282 * \ln(M_{head} + 1)$	0.91	<0.00001	<0.00001
Lollo	Cy3MG	$=0.46 + 0.000159 * CO_2 - 0.053 * \ln(M_{head} + 1)$	0.43	<0.00001	0.0004
	Q3MG	$=5.92 + 0.000529 * CO_2 - 0.979 * \ln(M_{head} + 1)$	0.80	0.001	<0.00001
	Q3Gc	$=1.91 + 0.000169 * CO_2 - 0.345 * \ln(M_{head} + 1)$	0.82	0.001	<0.00001
	L7Gc	$=0.20 + 0.000005 * CO_2 - 0.037 * \ln(M_{head} + 1)$	0.84	0.332	<0.00001
	Chicoric acid	$=23.62 + 0.00049 * CO_2 - 3.476 * \ln(M_{head} + 1)$	0.72	0.459	<0.00001
	Chlorogenic acid	$=3.63 + 0.000771 * CO_2 - 0.439 * \ln(M_{head} + 1)$	0.51	0.00001	<0.00001
	Caffeoylmalic acid	$=0.21 + 0.000218 * CO_2 + 0.060 * \ln(M_{head} + 1)$	0.53	<0.00001	0.003
	Caftaric acid	$=0.82 - 0.000029 * CO_2 - 0.091 * \ln(M_{head} + 1)$	0.58	0.252	<0.00001
	Isochlorogenic acid	$=0.42 + 0.000102 * CO_2 - 0.070 * \ln(M_{head} + 1)$	0.56	0.00001	<0.00001

(Carpenter, Keidel, Pihl, & Hughes, 2014; Gould, 2004). However, it does not seem to apply here. The observed increase in anthocyanin concentration in our experiment, is more likely to be related to high sugar concentrations as described in Section 3.3.

3.2.2. Caffeic acid derivatives

Results of the regression analysis are depicted in Fig. 3 with one curve per CO_2 concentration applied. Equations of the regression curves and their coefficients of determination as well as the *p*-values of the two involved factors are given in Table 1.

The concentration of chicoric acid was significantly higher in Oak Leaf lettuce cultivated at 1000 compared to 200 ppm CO_2 concentration. In Lollo, its concentration was not significantly influenced by the CO_2 concentration. In both cultivars, the concentration significantly decreased with increasing head mass. Chlorogenic acid concentrations were higher in 1000 compared to 200 ppm CO_2 concentration and decreased with increasing head mass, in both cultivars. Caffeoylmalic acid concentration in Oak Leaf was neither influenced by the CO_2 concentration nor by head mass. In Lollo, caffeoylmalic acid concentration was higher in plants cultivated at 1000 compared to 200 ppm CO_2 concentration and, remarkably, increased with increasing head mass. In both cultivars, the concentration of caftaric acid was not influenced by CO_2 concentration but decreased with increasing head mass. Isochlorogenic acid concentrations were higher in plants cultivated at 1000 compared to 200 ppm CO_2 concentration and decreased with increasing head mass. Hence, in both cultivars 3 out of 5 caffeic acid derivatives were positively influenced by increasing CO_2 concentration which is in line with the results of Peltonen et al. (2005) obtained on birch. To our knowledge, the response of single foliar phenolic acids to elevated CO_2 concentrations has not been studied before. However, they are contributing to the total phenolics concentration which was also observed to increases in response to elevated CO_2 concentration in wheat leaves but not in orange or pine trees (Peñuelas et al., 1996). The variance among the single caffeic acid derivatives we detected in our experiment underlines the significance of detailed measurements, preferably using HPLC, instead of total phenolics.

The decreasing concentrations of most caffeic acid derivatives with increasing head mass are in line with previous results (Becker et al., 2014b).

Chicoric, chlorogenic, caffeoylmalic, caftaric and isochlorogenic acid resemble each other in their structure. Caftaric acid is an ester of caffeic acid and tartaric acid while chicoric acid comprises one more caffeoyl moiety. Chlorogenic acid is an ester of caffeic acid and quinic acid while isochlorogenic acid comprises one more caffeoyl moiety. Caffeoylmalic acid is an ester of one caffeic acid and one malic acid. In Lollo, chicoric and caftaric acid showed a uniform response to CO_2 concentration and plant age, as did chlorogenic and isochlorogenic acid. In Oak Leaf, only the latter showed a uniform response. Nevertheless, the acid bound to the caffeic acid moiety seems to be more influential on the response regarding CO_2 concentration and head mass than the number of caffeic acid moieties in the molecule: We detected three caffeic acid derivatives comprising only one caffeic acid moiety but their responses to the studied factors resembled each other less than that of those with either tartaric or quinic acid.

The response of caffeoylmalic acid is unlike the other caffeic acid derivatives. In Oak Leaf, the factors we studied were obviously not of influence. In Lollo, its concentration increased with plant age. We did not observe a similar response with any of the other phenolic compounds.

Except for their ability to absorb ultraviolet radiation and their antioxidant activity, not much is known about the function of caffeic acid derivatives and neither of these characteristics is likely to account for the observed effects in our experiment.

3.3. Sugar concentrations

Results of the regression analysis are depicted in Fig. 4, with one curve per CO_2 concentration applied. Equations of the regression curves and their coefficients of determination as well as the *p*-values of the two involved factors are given in Table 2. In both cultivars, sugar concentrations were significantly higher in plants cultivated at 1000 compared to 200 ppm CO_2 concentration. The response of sucrose, glucose, and fructose concentrations was uniform within each cultivar (Fig. 4).

Head mass had a significant influence on sugar concentration regarding Lollo but not regarding Oak Leaf lettuce (Table 2). In our experiment, high flavonoid concentrations coincide with high sugar concentrations. This is in line with the hypothesis that high CO_2 concentrations increase precursor availability for flavonoid

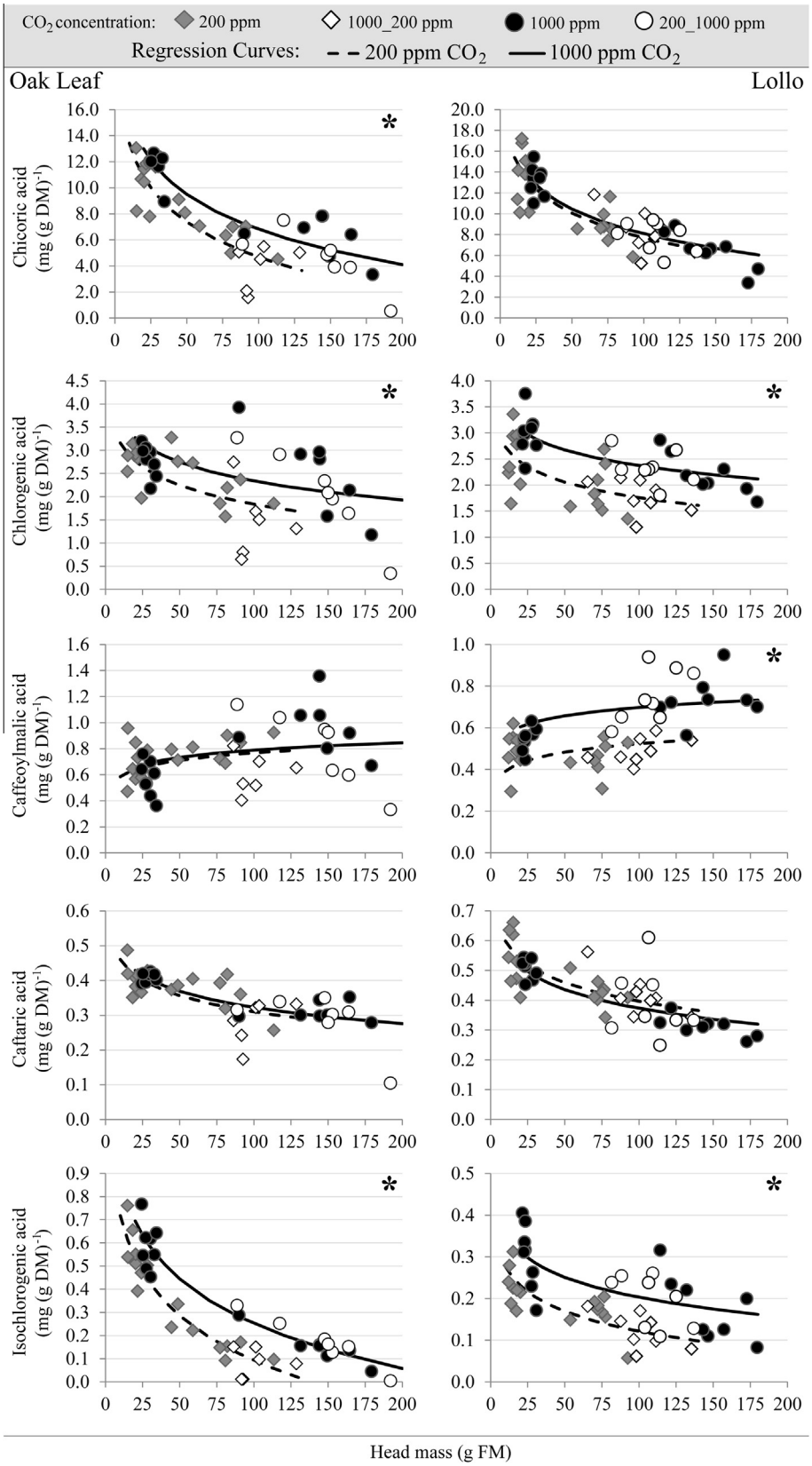

Fig. 3. Effect of CO_2 concentration (ppm) and head mass (g fresh matter) on the concentration of caffeic acid derivatives per gram dry matter (g DM) in two red leaf lettuce cultivars (Oak Leaf and Lollo). Grey symbols represent plants cultivated at CO_2 concentrations of 200 ppm, black symbols represent plants cultivated at 1000 ppm all the time. White symbols represent plants that have been exchanged between treatments and thus been cultivated first at 1000 then at 200 ppm (diamonds) or first at 200 then at 1000 ppm (circles). The effect of CO_2 concentration and head mass on caffeic acid derivative concentrations was evaluated via multiple regression analysis (equations in Table 1). The solid and broken lines depict the caffeic acid derivative concentrations calculated with the regression equation for 1000 and 200 ppm CO_2 concentration, respectively. Asterisks mark significant differences between the CO_2 treatments.

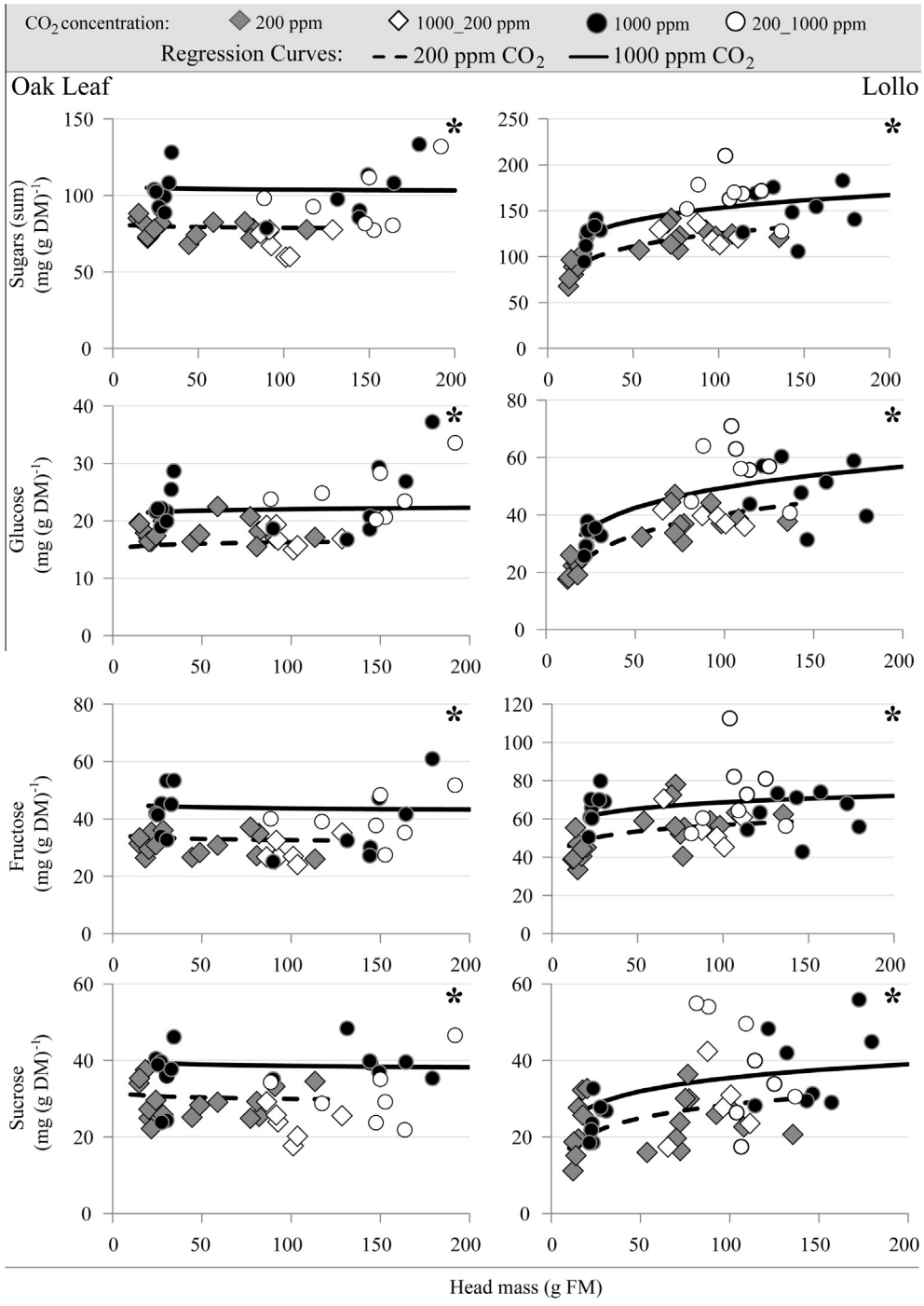

Fig. 4. Effect of CO_2 concentration (ppm) and head mass (g fresh matter) on the concentration of sugars per gram dry matter (g DM) in two red leaf lettuce cultivars (Oak Leaf and Lollo). Grey symbols represent plants cultivated at CO_2 concentrations of 200 ppm, black symbols represent plants cultivated at 1000 ppm all the time. White symbols represent plants that have been exchanged between treatments and thus been cultivated first at 1000 then at 200 ppm (diamonds) or first at 200 then at 1000 ppm (circles). The effect of CO_2 concentration and head mass on sugar concentrations was evaluated via multiple regression analysis. The solid and broken lines depict the sugar concentrations calculated with the regression equation for 1000 and 200 ppm CO_2 concentration, respectively. Asterisks mark significant differences between the CO_2 treatments.

biosynthesis which results in high flavonoid concentrations: Zhang et al. (2013) proposed that carbohydrates trigger anthocyanin biosynthesis in *Begonia semperflorens*. They suggested excess carbohydrates to be the proximate trigger of leaves reddening in autumn when carbohydrates are accumulated for storage. Sucrose is reported to directly induce anthocyanin biosynthesis in *A. thaliana* (Solfanelli et al., 2006). Glucose and fructose can feed the pentose phosphate pathway which provides erythrose-4-phosphate, a precursor for the phenylalanine producing shikimate pathway, eventually leading to flavonoid aglycone biosynthesis (Jaafar et al., 2012; Schopfer & Brennicke, 2010). Additionally, another glucose molecule is consumed to form glycosides.

Flavonols, flavones, caffeic acid derivatives share various biosynthetic steps with anthocyanins. Precursor abundancy may therefore well have had the same effect on their biosynthesis like hypothesized for anthocyanins.

Table 2
Effects of CO_2 concentration during the 10 days before harvest (CO_2, ppm) and head mass at harvest (M_{head}, g) on the concentration of sugars (mg (g DM)$^{-1}$). Coefficients were estimated using quasilinear regression analysis separately for each cultivar based on all samples harvested 11 and 25 DAP. R^2 denotes the coefficient of determination.

Cultivar	Compound	Equation of regression curve	R^2	p-Value for	
				CO_2	M_{head}
Oak Leaf	Total sugars	$=76.1 + 0.0312 * CO_2 - 1.74 * \ln(M_{head} + 1)$	0.49	<0.00001	0.500
	Glucose	$=13.2 + 0.00695 * CO_2 + 0.83 * \ln(M_{head} + 1)$	0.40	0.00005	0.283
	Fructose	$=32.6 + 0.0138 * CO_2 - 1.36 * \ln(M_{head} + 1)$	0.36	0.00002	0.357
	Sucrose	$=30.3 + 0.0105 * CO_2 - 1.22 * \ln(M_{head} + 1)$	0.30	0.00016	0.344
Lollo	Total sugars	$=23.8 + 0.0346 * CO_2 + 20.5 * \ln(M_{head} + 1)$	0.66	<0.00001	<0.00001
	Glucose	$=-10.4 + 0.0112 * CO_2 + 10.5 * \ln(M_{head} + 1)$	0.40	0.00028	<0.00001
	Fructose	$=31.2 + 0.0147 * CO_2 + 4.93 * \ln(M_{head} + 1)$	0.31	0.00232	0.0326
	Sucrose	$=2.98 + 0.00873 * CO_2 + 5.15 * \ln(M_{head} + 1)$	0.32	0.0140	0.00361

Yet, incorporating carbon into phenolic compounds in times of plenty appears to be a one-way-street. There is no mechanism known how plants could retrieve these carbon atoms in meager times.

4. Summary and conclusions

The results partly confirm our first hypothesis that high CO_2 concentration has a positive effect on the concentration of flavonoid glycosides, except for luteolin-7-*O*-glucuronide in red Lollo, and on 3 out of 5 caffeic acid derivatives, but different ones in each cultivar. We could fully confirm our second hypothesis: the CO_2 concentration shortly before harvest determines the concentration of flavonoid glycosides and caffeic acid derivatives. Additionally, our results support the third hypothesis that high CO_2 concentrations increase the availability of precursors for the biosynthesis of phenolic compounds. From a practical viewpoint, this shows the relevance of CO_2 enrichment of the atmosphere in greenhouses and plant factories in order to increase the concentration of health promoting phenolic compounds in lettuce. Ecologically interpreted, however, we were not able to explain our results with existing theories on the antioxidative or photoprotective mode of action of flavonoids and caffeic acid derivatives in plants. They may rather act as sinks for copious amounts of photosynthates. More detailed research into lettuce physiology is necessary here.

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