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Cool-cultivated red leaf lettuce accumulates cyanidin -3-*O*-(6″-*O*-malonyl)-glucoside and caffeoylmalic acid

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ABSTRACT

Cultivating lettuce in greenhouses at low temperatures improves its CO_2-balance and may increase its content of flavonoid glycosides and phenolic acids. We cultivated 5 weeks old red leaf lettuce seedlings at 20/15 °C (day/night) or 12/7 °C until plants reached comparable growth stages: small heads were harvested after 13 (warm) and 26 (cool) days, while mature heads were harvested after 26 (warm) or 52 (cool) days. Additionally, some plants were cultivated first cool then warm and vice versa (39 days). Cool-cultivated small heads had higher concentrations of cyanidin-3-*O*-(6″-*O*-malonyl)-glucoside and caffeoylmalic acid than warm-cultivated ones but we detected no differences concerning quercetin and luteolin glycosides or di-*O*-caffeoyltartaric and 5-*O*-caffeoylquinic acid. Regarding mature heads, there were only differences concerning cyanidin-3-*O*-(6″-*O*-malonyl)-glucoside. We therefore suggest that only cyanidin-3-*O*-(6″-*O*-malonyl)-glucoside was truly responsive to temperatures alone. Previously reported contrasting effects may rather be due to comparison of different growth stages or interactive effects with radiation.

1. Introduction

Epidemiological studies associate a diet rich in polyphenols with lower incidence of coronary heart disease or cancer (Cartea, Francisco, Soengas, & Velasco, 2011). Red leaf lettuce (*Lactuca sativa* L.) is an increasingly important crop and a good dietary source of polyphenols as it contains several phenolic acids (caffeic acid derivatives) and flavonoid (quercetin, luteolin and cyanidin) glycosides (Llorach, Martínez-Sánchez, Tomás-Barberán, Gil, & Ferreres, 2008). Though lettuce is not the vegetable richest in polyphenols, it still provides for a considerable amount of daily polyphenol intake because it is commonly consumed raw and in large quantities (DuPont, Mondin, Williamson, & Price, 2000). It is, for example, one of the main sources of chicoric and caffeoylmalic acid in the Central European diet (Clifford, 2000). The major phenolic compounds in red leaf lettuce are quercetin-3-*O*-glucoside, quercetin-3-*O*-(6″-*O*-malonyl)-glucoside, quercetin-3-*O*-glucuronide, luteolin-7-*O*-glucuronide and cyanidin 3-*O*-(6″-*O*-malonyl)-glucoside as well as di-*O*-caffeoyl tartaric acid (chicoric acid), 5-*O*-caffeoylquinic acid (chlorogenic acid) and *O*-caffeoylmalic acid (Llorach et al., 2008). Several of these substances have been ascribed antioxidative and antiatherogenic effects as well as inhibitive effects on lipid peroxidation and cyclooxigenase enzymes (Cartea et al., 2011).

In the cool seasons in Central Europe, lettuce is usually cultivated in greenhouses which tend to consume large amounts of energy – mostly derived from fossil fuels. Due to economic and ecological reasons, strategies to improve greenhouse CO_2-balances are currently being developed. One approach to save energy for heating is to cultivate crops at lower temperatures. This influences plants in manifold ways: Decreasing temperature generally slows down metabolic processes. With lettuce, this results for example in delayed growth, hence postponed development of marketable lettuce heads (Wurr, Fellows, & Phelps, 1996), while it is also very likely to influence quality parameters like secondary metabolites (Treutter, 2010). Concerning flavonoids, there are indications that biosynthesis increases with lower temperatures (Harbaum-Piayda et al., 2010; Havaux & Kloppstech, 2001; Neugart et al., 2012). However, there are only few studies on the effect of temperature on the phenolic compounds in lettuce (Boo, Heo, Gorinstein, & Chon, 2011; Gazula, Kleinhenz, Streeter, & Miller, 2005; Oh, Carey, & Rajashekar, 2009).

In plants, the general deceleration of metabolic processes at low temperature affects for example the Calvin cycle enzymes of the light-independent part of photosynthesis (Havaux & Kloppstech, 2001). Thus, the intercepted light may eventually become over-excessive and lead to the formation of reactive oxygen species

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(ROS) by leakage of energy and/or electrons to molecular oxygen (Havaux & Kloppstech, 2001). ROS have the potential to destroy thylakoid membranes (the site of the light-dependent photosynthetic reactions), damage DNA, and denature proteins (Gould, Neill, & Vogelmann, 2002). The detrimental effects of low temperature-induced oxidative damage are enforced by the fact that also enzymatic repair processes are slowed down. However, ROS themselves can be perceived by plants. They can act as messenger molecules, eventually influencing gene expression and conveying acclimation to an altered environment (Edreva, 2005; Gill & Tuteja, 2010).

Flavonoids are well known for their antioxidant properties, especially those comprising an *ortho* 3′,4′-dihydroxy moiety in the B ring like quercetin, luteolin and cyanidin (Rice-Evans, Miller, & Paganga, 1997). Anthocyanins additionally function as photoprotectant by absorbing part of incident light (Gould et al., 2002). Interestingly, transcription factors of flavonoid biosynthesis have been reported to be influenced by changes of the plant cell redox potential (Agati & Tattini, 2010).

Data on the response of phenolic acid biosynthesis to low temperatures is less consistent. Some studies report increasing phenolic acid concentration with low temperatures (Zidorn, 2010), some find no effect of temperature alone but rather in combination with other factors like radiation intensity or nitrogen supply (Grace, Logan, & Adams, 1998; Løvdal, Olsen, Slimestad, Verheul, & Lillo, 2010) while others find different phenolic acids to respond differently (Oh et al., 2009). Clearly, more and attentive research is needed here.

To the best of our knowledge, there is no study on the long term effect of low temperature on the major phenolic compounds in red leaf lettuce: Oh et al. (2009) only applied low temperatures for 1 day. Gazula et al. (2005) subjected plants to temperature treatments for 20 days but investigated only the accumulation of anthocyanins and in a higher temperature range (20–30 °C). Boo et al. (2011) cultivated plants for 6 weeks but only measured anthocyanins and total polyphenols. Furthermore, they did not take into account that together with varying temperature, the plants' growth rates vary (Wurr et al., 1996). Data published by Romani et al. (2002) suggest higher concentrations of quercetin glycosides and phenolic acids in lettuce in early growth stages compared to later ones. The relevance of head development for the concentration of quercetin glycosides has also been reported for other vegetables (Krumbein, Saeger-Fink, & Schonhof, 2007).

Therefore, in this study we implemented a new approach and determined the harvest dates based on the concept of accumulated thermal time instead of elapsed time (Tei, Aikman, & Scaife, 1996). We composed a harvest schedule that allowed us, on the one hand, to obtain information on plants in comparable growth stages which they reached after a different number of days due to differing temperature regimes (Tei et al., 1996; Wurr et al., 1996), in order to try and exclude developmental effects from our analysis and to obtain marketable lettuce heads in every treatment to gain results of practical relevance. On the other hand, we harvested lettuce plants cultivated at different temperature after the same number of days in order to compare results to previous studies. Furthermore, we tested the influence of low temperature in an early and in a more advanced growth stage, additionally to exposing plants continuously to either the cool or the warm temperature regime, because the effect of temperature can vary during ontogeny (Wheeler, Hadley, Ellis, & Morison, 1993). We chose red leaf lettuce for its rich phenolic profile. As cultivars can differ distinctly, we included two cultivars in the experiment. For each treatment, cultivar, and replicate, we measured the concentration of flavonoid glycosides and phenolic acids, assessed head mass, number of leaves, and dry matter content.

To sum up, we wanted to investigate three hypotheses with this experiment:

(I) Cool-cultivated lettuce plants contain higher concentrations of phenolic compounds than warm-cultivated ones.
(II) Different phenolic compounds in red leaf lettuce vary in their response to low temperature.
(III) Small lettuce plants are influenced differently by temperature than mature heads.

Experiments were conducted in growth chambers to strictly separate the effects of temperature from radiation because they are known to strongly interact (Løvdal et al., 2010).

2. Material and methods

2.1. Plant cultivation

Red Oak Leaf and red Lollo lettuce (*L. sativa* L. var. *crispa* L. cv. Eventai RZ and *L. sativa* L. var. *crispa* L., cv. Satine, respectively; RijkZwaan, De Lier, The Netherlands) differ regarding their recommended greenhouse cultivation schedule: The seed company recommends red Oak Leaf from fall to spring, throughout the winter (November to April), while for Lollo Rosso cultivation in late fall and spring is advised.

The seeds were sown in rockwool cubes, kept at 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 (5 weeks old) and weighed about 0.9 g they were transferred into growth chambers (Yorck, Mannheim, Germany) where they were grown using deep flow technique, in four growth chambers simultaneously. The nutrient solution was prepared according to Sonneveld and Straver (1988) and exchanged and analyzed every week. In two chambers, the air temperature was 20 °C during daytime and 15 °C at night (warm treatment), whereas it was 12/7 °C (day/night) in the other two (cool treatment). Relative humidity was approximately 80%. Radiation was supplied by high-pressure sodium discharge lamps SON-T PLUS 400 W (Philips, Amsterdam, The Netherlands). 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 247 μmol m^{-2} s^{-1}, during dusk and dawn, respectively, only some of the lamps were switched on, resulting in a mean PPFD of 95 μmol m^{-2} s^{-1}, as measured with a portable light meter Li-250 (Li-COR Inc., Lincoln, Nebraska, USA). Hence, the plants intercepted a daily light integral of 11.4 mol m^{-2} - day^{-1}. Plants cultivated for 13 days intercepted a total light integral of 148 mol photosynthetically active radiation (PAR), while those cultivated for 26, 39 and 52 days intercepted 296, 445, and 593 mol PAR m^{-2} s^{-1}, respectively.

To elucidate harvest dates at which the plants cultivated in different temperatures will have reached a comparable growth stage (based on head mass and number of leaves) we used the concept of "sum of temperatures". As rates of metabolic processes are temperature dependent, this concept uses the accumulated thermal time instead of elapsed time to predict plant growth and development (Tei et al., 1996). Accumulated thermal time is measured in day-degrees (DD). It is calculated by adding the values for daily mean temperature. This concept is widely used in horticultural crop production to predict harvest dates and decide when to sow and plant. Based on previous experiments (data not shown), we set a target value of 400 DD (starting on the day of transfer into growth chamber) to obtain marketable lettuce heads of 200–250 g at the end of this experiment. Most crops have a "base temperature" below which no growth occurs. Based on previous experiments, we assumed a base temperature of 2 °C which is subtracted from the daily mean temperature in the calculations. The warm treatment reached the set day-degrees 26 days after planting

(406 DD), the cool treatment 52 days after planting (395 DD). Some plants were exchanged after they reached half of the day-degrees (203 and 198 DD, after 13 and 26 days in the warm and cool treatment, respectively) and harvested after 39 days. On day 13 and 26 after planting, some plants were harvested from the warm and the cool treatment. Thus, at the end we had information about lettuce plants from the following six conditions and stages: small heads grown warm or cool (ca. 200 DD), as well as mature heads cultivated warm, cool, first cool then warm and first warm then cool (ca. 400 DD; see harvest schedule, Fig. 1).

2.2. Plant growth characteristics

For all samples, only above ground organs (lettuce heads) were harvested. At all harvest dates, three heads per cultivar, treatment, and replicate were weighed to obtain the mean head mass. Values for head mass are given in gram fresh matter (FM). To obtain dry matter content, weight before and after lyophilization was compared. Values for dry matter content are given as milligram dry matter per gram fresh matter.

2.3. Sample preparation

A mixed sample from six heads was prepared for each cultivar, treatment, and replicate only limp or deteriorated outer leaves were removed. Within 30 min after harvesting, the plants were cut in smaller pieces, mixed and 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).

2.4. Analyses of phenolic compounds

The well-established HPLC-DAD-ESI-MS method for the determination of flavonol glycosides and phenolic acids in kale, reported by (Neugart et al., 2012) was optimized for lettuce. Best results were obtained by extracting 0.5 g of lyophilized, pulverized lettuce powder with 25 ml of aqueous methanol (50% MeOH) at room temperature. The suspension was kept in motion with a magnetic stirrer for 1.5 h and then centrifuged (Labofuge 400R, Heraeus Instruments, Thermo Fisher Scientific, Waltham, USA) for 15 min at 4500 rcf (relative centrifugal force). The supernatant was filtered with PTFE-syringe filters (0.25 µm, polytetrafluoroethylene; Roth, Karlsruhe, Germany) transferred to a glass vial and analyzed via HPLC-DAD-ESI-MS^3.

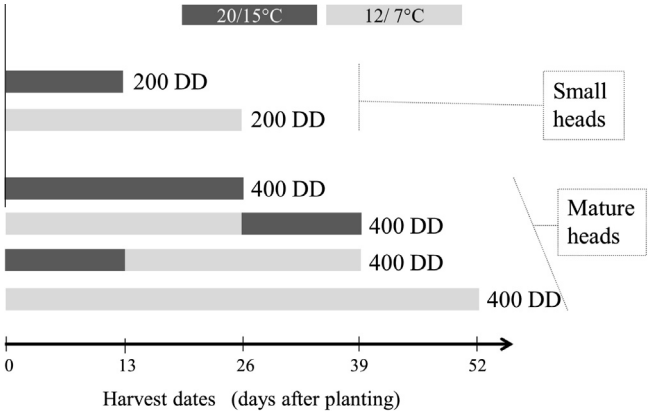

Fig. 1. Harvest schedule based on accumulated thermal time, measured in day-degrees (DD). Dark grey bars represent warm cultivation at 20/15 °C (day/night), light grey bar represent cool cultivation at 7/12 °C. Plants in the warm temperature regime reached the day-degrees set for harvest earlier than the plants in the cool regime. The target value for harvesting mature, marketable lettuce heads of 200–250 g was 400 DD. Some plants were exchanged between the warm and the cool growth chambers after they reached half of the day-degrees aimed for (200 DD; 13 and 26 days after planting with warm- and cool-cultivated plants, respectively), in order to study the influence of temperature on lettuce in different growth stages. We obtained two and four variants, respectively: small heads cultivated either warm or cool as well as mature heads cultivated cool, warm, and first cool then warm or vice versa. Thus we were able to, on the one hand, compare them in corresponding growth stages and on the other hand compare cool- and warm-cultivated plants after the same number of days (26).

The anthocyanin extracts were prepared similarly to the method applied to flavonols, 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 15 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 Prodigy column (ODS 3, 150 × 3 mm, 5 µm, 100 Å; Phenomenex, Aschaffenburg, Germany) with a security guard C18 (ODS 3, 4 × 3 mm, 5 µm, 100 Å) 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: 7–9% (10 min), 9–12% (20 min), 12–15% (55 min), 15–50% (5 min), 50% isocratic (5 min), 50–7% (5 min), and isocratic 7% (3 min). Gradient 2 was distinctly shorter: 10–50% B (10 min), 50% B isocratic (10 min), 50–10% B (5 min) and 10% B isocratic (5 min). Flow rate in both gradients was 0.4 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*. However, anthocyanin 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 et al., 2000; Llorach et al., 2008). Standard substances were purchased at Carl Roth GmbH (Karlsruhe, Germany; quercetin-3-*O*-glucoside, 5-*O*-caffeoylquinic acid) and Sigma–Aldrich GmbH (Munich, Germany; quercetin-3-glucuronide, di-*O*-caffeoyltartaric acid, cyanidin-3-*O*-glucoside).

The DAD was used for quantification, using the detection wavelengths 330 nm (phenolic acids), 350 nm (flavonol glycosides) and 520 nm (anthocyanin glycosides). External calibration curves were prepared in the respective relevant concentrations, using the standard substances where available. Cyanidin and quercetin-3-*O*-malonylglucosides were quantified as their respective 3-*O*-glucoside equivalents. Caffeoylmalic acid is presented as 5-*O*-caffeoylquinic acid equivalents.

2.5. Statistical analyses

In order to detect significant differences induced by the different temperature regimes, two-way ANOVA was performed (Fisher's *F*-test) followed by Tukey's Honest Significant Difference test. A significance level of $\alpha = 0.05$ was used. Calculations were performed using R statistics (version 2.10.1 ed., R Development Core Team, Vienna, Austria).

3. Results and discussion

3.1. Influence of cultivar on growth and phenolic compounds

The two cultivars of red leaf lettuce showed significant quantitative but no qualitative differences regarding most of the phenolic compounds and growth parameters (Table 1). In detail, head mass and dry matter content were higher with red Oak Leaf than with Lollo Rosso lettuce, whereas the concentrations of cyanidin, quercetin and luteolin glycosides, as well as of chicoric and chlorogenic acid, were higher in Lollo Rosso than in red Oak Leaf lettuce (data not shown). This is in line with previous studies (Llorach et al., 2008). We detected no interactions between temperature treatment and lettuce cultivar (Table 1). In the following, we therefore display the average effect of the temperature treatments on both cultivars.

3.2. Influence of temperature on head mass, number of leaves, and dry matter content

Plants harvested after 200 DD had a mean head mass of 42.8 ± 13.7 g and will be further referred to as "small heads" while plants harvested after 400 DD, with a mean head mass of 242.9 ± 35.5 g, will be referred to as "mature heads".

Small heads that were cultivated cool for 26 days had a significantly higher mass than small heads cultivated warm for 13 days (Fig. 2 and Table 1). Also regarding mature heads, cool-cultivated plants had a significantly higher head mass than warm-cultivated ones, while head mass of plants that had been transferred between temperature regimes lay in between (Fig. 2).

Generally, lettuce heads were heavier the more days they were cultivated. This can be explained by the different total light integrals the plants experienced (see Section 2.1).

Table 1
The influence of temperature and cultivar on the concentration of growth characteristics and phenolic compounds, assessed by two-way ANOVA (*F*-test; factor 1, treatment; factor 2, cultivar; $n = 2$). Data was evaluated separately for the two growth stages investigated. Q3G, quercetin-3-*O*-glucoside; Q3MG, quercetin-3-*O*-(6″-*O*-malonyl)-glucoside; Q3Gc/L7Gc, quercetin-3-*O*-glucuronide/luteolin-7-*O*-glucuronide; Cy3MG, cyanidin-3-*O*-(6″-*O*-malonyl)-glucoside. The given *p*-values display the probability that the observed differences occurred by chance.

Characteristics	Growth stage	*p*-Values		
		Temperature	Cultivar	Interaction
Plant growth				
Head mass	Small heads	0.001	0.002	0.13
	Mature heads	<0.0001	<0.0001	0.17
Number of leaves	Small heads	0.07	0.002	0.19
	Mature heads	0.002	<0.0001	0.87
Dry matter content	Small heads	0.045	0.003	0.26
	Mature heads	0.009	0.003	0.52
Anthocyanidin glycoside				
Cy3MG	Small heads	0.04	0.06	0.19
	Mature heads	0.02	<0.0001	0.23
Flavonol and flavone glycosides				
Q3G	Small heads	0.73	0.16	0.42
	Mature heads	0.84	0.02	0.92
Q3MG	Small heads	0.44	0.02	0.45
	Mature heads	0.79	0.003	0.92
Q3Gc/L7Gc	Small heads	0.13	0.02	0.23
	Mature heads	0.81	0.003	0.86
Phenolic acids				
Chicoric acid	Small heads	0.84	0.006	0.75
	Mature heads	0.78	0.03	0.89
Chlorogenic acid	Small heads	0.11	0.03	0.94
	Mature heads	0.70	0.05	0.88
Caffeoylmalic acid	Small heads	0.004	0.89	0.46
	Mature heads	0.27	0.49	0.76

Small heads had a mean number of leaves of 18.1 ± 1.5, without significant differences between warm- and cool-cultivated ones (Fig. 2 and Table 1). Mature heads on average developed 39.4 ± 4.4 leaves per plant, with significant differences between plants from different treatments: Plants cultivated cool all the time or only for the first weeks had a significantly higher number of leaves than plants cultivated warm for the first weeks or all the time (Fig. 2 and Table 1). Obviously, the temperature regime in earlier growth stages determined the number of leaves the mature heads developed.

Cool-cultivated small heads had a higher dry matter content than warm cultivated ones (Fig. 2 and Table 1). Cool-cultivated mature heads, as well as those that had been transferred from warm to cool, had a higher dry matter content than warm-cultivated ones, while that of plants which had been transferred from cool to warm was in between (Fig. 2 and Table 1). In general, differences between small heads and mature heads were not as pronounced as regarding head mass (Fig. 2), although small heads on average had higher dry matter content than mature heads (5.6% and 4.7%, respectively).

Previous studies (Boo et al., 2011) compared plants' phenolic content after having subjected them to different temperatures for the same number of days. Therefore, we also compared the growth of plants cultivated cool and warm for 26 days (Table 2). Plants that have been cultivated warm for 26 days had a much higher head mass (194.1 g FM) and number of leaves (34.7) than those cool cultivated for 26 days (52.5 g FM, 19.2; Fig. 2 and Table 2) indicating that they are in a more advanced growth stage than cool-cultivated ones. These differences are much more pronounced than between small heads or between mature heads (Fig. 2 and Tables 1 and 2). Additionally, after 26 days, dry matter content was higher in cool- than in warm-cultivated plants (5.8%, 4.1%; Fig. 2 and Table 2). Obviously, the growth characteristics differ strongly between plants cool- and warm-cultivated for 26 days.

We want to emphasize that the differences between plants harvested after approximately the same day-degrees were much smaller. Thus, in order to single out the effect of temperature alone and to obtain results of practical relevance, we considered it more meaningful to compare plants in corresponding growth stages.

3.3. Influence of temperature on flavonoid glycosides

In our HPLC-DAD-ESI-MS^3 analyses of flavonol, flavone and anthocyanidin glycosides as well as phenolic acids 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 and cyanidin-3-*O*-(6″-*O*-malonyl)-glucoside, quercetin-3-*O*-glucuronide and 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 (Becker, Kläring, Kroh, & Krumbein, 2013; DuPont et al., 2000; Llorach et al., 2008; Romani et al., 2002). Quercetin-3-*O*-glucuronide and luteolin-7-*O*-glucuronide co-eluted and were quantified as sum. Mass spectrometric data suggested they in average contributed in equal shares to the peak evaluated via DAD which is in line with data obtained by DuPont et al. (2000).

3.3.1. Anthocyanidin glycoside

The concentration of cyanidin-3-*O*-(6″-*O*-malonyl)-glucoside was significantly higher in cool-cultivated than in warm-cultivated small heads (Fig. 3 and Table 1). In mature heads, the first warm- then cool-cultivated plants had the highest mean concentration of cyanidin glycosides, significantly higher than plants cultivated first cool then warm (Fig. 3 and Table 1). Regarding mature heads,

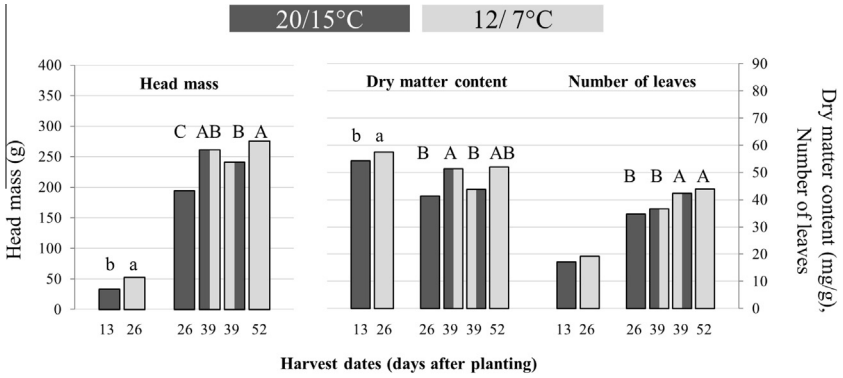

Fig. 2. Head mass in gram fresh matter (FM), dry matter content (milligram per gram FM) and number of leaves of red leaf lettuce, cultivated in different temperature regimes for a different number of days. Light-grey bars represent cultivation at 12/7 °C, dark grey bars represent cultivation at 20/15 °C. For detailed description of the treatments, please see caption of Fig. 1. Identical letters on top of bars show that these treatments do not differ significantly ($n = 2$; Tukey-test, $\alpha = 0.05$).

Table 2
Influence of temperature and cultivar on concentration of growth characteristics and phenolic compounds after cultivation at 12/7 °C and 20/15 °C (day/night) for 26 days, assessed by two-way ANOVA (*F*-test; factor 1, growth stage; factor 2, cultivar; $n = 2$). Q3G, quercetin-3-*O*-glucoside; Q3MG, quercetin-3-*O*-(6″-*O*-malonyl)-glucoside; Q3Gc/L7Gc, quercetin-3-*O*-glucuronide/luteolin-7-*O*-glucuronide; Cy3MG, cyanidin-3-*O*-(6″-*O*-malonyl)-glucoside. The given *p*-values display the probability that the observed differences occurred by chance.

Characteristics	*p*-Values		
	Temperature	Cultivar	Interaction
Plant growth			
Head mass	<0.0001	0.002	0.01
Number of leaves	<0.001	0.001	0.30
Dry matter content	0.01	0.04	0.65
Anthocyanidin glycoside			
Cy3MG	0.02	0.02	0.40
Flavonol and flavone glycosides			
Q3G	0.10	0.05	0.44
Q3MG	0.009	0.01	0.21
Q3Gc/L7Gc	0.005	0.01	0.11
Phenolic acids			
Chicoric acid	0.01	0.03	0.50
Chlorogenic acid	0.11	0.11	0.83
Caffeoylmalic acid	0.01	0.67	0.95

there is no significant difference between plants cultivated warm or cool all the time (Fig. 3 and Table 1).

Boo et al. (2011) reported elevated anthocyanin concentration in lettuce due to low temperature. In their experiment, lettuce was grown for the same number of days (6 weeks) at temperatures as diverse as 30/25 °C and 13/10 °C. Plants from these treatments probably differed strongly regarding their growth stages (see Section 3.2 for comparison). If we compare plants cultivated cool or warm for the same number of days in our experiment, we also detect significant differences (26 days; Table 2 and Fig. 3). However, we additionally detected significantly higher anthocyanin concentration in cool-cultivated plants when we compared them to warm-cultivated plants in a corresponding growth stage for small heads (Table 1 and Fig. 3).

Nevertheless, this accumulation in cool-cultivated small head seems to only have been transient: As mature heads, cool-cultivated plants have a much lower anthocyanin concentration than as small heads. Small heads that had been subjected to low temperature had a 59% higher anthocyanin concentration than warm-cultivated small heads. Regarding mature heads, first warm- than cool-cultivated plants only had a 17% higher anthocyanin concentration than the corresponding warm-cultivated plants. The first mentioned difference was significant while the latter was

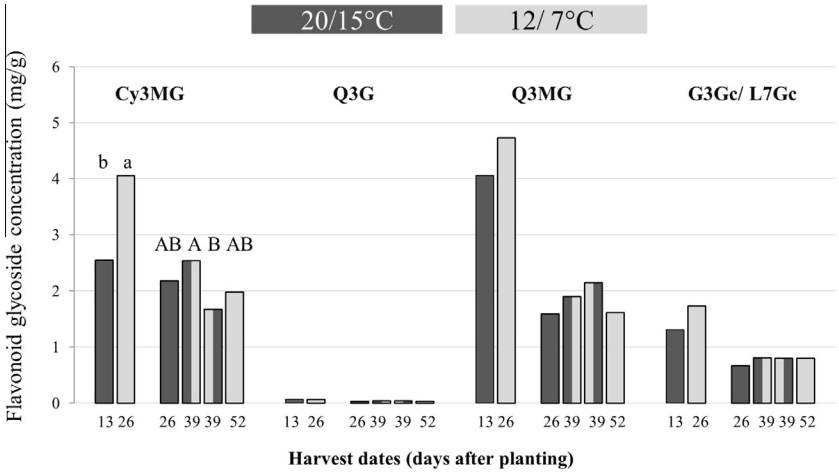

Fig. 3. Concentration of flavonoid glycosides related to dry matter (DM) of red leaf lettuce, cultivated in different temperature regimes for a different number of days. Cy3MG, cyanidin-3-*O*-(6″-*O*-malonyl)-glucoside; Q3G,Assuming a connection between ROS production quercetin-3-*O*-glucoside; Q3MG, quercetin-3-*O*-(6″-*O*-malonyl)-glucoside; Q3Gc/L7Gc, quercetin-3-*O*-glucuronide/luteolin-7-*O*-glucuronide. For each compound, the first two bars represent small lettuce heads (days 13 and 26), while the other four bars (days 26, 39, 39, and 52) represent mature heads. For detailed description of the treatments, please see caption of Fig. 1. Identical letters on top of bars show that these treatments do not differ significantly ($n = 2$; Tukey-test, $\alpha = 0.05$).

not (Table 1). This indicates that the low temperature regime was more stressful to plants in an early than in a later growth stage.

When temperature is low, the light intercepted by plants and supplied to the electron transport chain of the photosynthetic apparatus in chloroplast thylakoid membranes may eventually become over-excessive because the enzymatic part of photosynthesis is slowed down. This may lead to over-reduction of the electron carriers, over-excitation of the photosystems, and eventually to the formation of ROS (Edreva, 2005; Havaux & Kloppstech, 2001). Neill and Gould (2003) suggest that cyanidin-3-*O*-(6″-*O*-malonyl)-glucoside acts as both antioxidant and light attenuator in Lollo Rosso lettuce: Accumulation of cyanidin glycoside in epidermal cell vacuoles can alleviate the oxidative load in photosynthetically active cells by absorbing part of the surplus photons that would otherwise be funnelled into the electron transport chains and possibly produce ROS. On the other hand, they can act as antioxidants in the cytosol of photosynthetic active cells and counteract ROS formation (Neill & Gould, 2003). According to Edreva (2005) different components of the photosynthetic apparatus produce different types of ROS when over-excited- superoxide anion radicals ($O_2^{\cdot-}$) being the "energy outlet" of the electron transport chain in chloroplasts. Cyanidin-3-*O*-(6″-*O*-malonyl)-glucoside is a very effective scavenger of $O_2^{\cdot-}$ (Neill & Gould, 2003).

Assuming a connection between ROS production by over-excited electron transport chains and anthocyanin accumulation, this would imply a lower oxidative load in cells of mature heads than in small heads, in our experiment. The reason for this may lie in their head architecture: The small heads had only developed 4 true leaves when subjected to low temperature while the larger ones already had 17 leaves and head formation had started. With advanced head formation, more and more leaves are shading each other, i.e. larger percentages of biomass are shielded from direct light. In these leaves less energy is funneled into the electron transport chain and less ROS are formed. The higher incidence of self-shading in mature heads would, therefore, result in a smaller number of leaves with potentially enhanced oxidative load per head, i.e. would explain the overall lower concentration of anthocyanins per head. Consistently, it has been established that inner leaves of lettuce heads have lower concentrations of flavonols than outer leaves- not due to a lack of competence but due to lower incident radiation intensity compared to the situation with outer leaves (Hohl, Neubert, Pforte, Schonhof, & Böhm, 2001).

The observation that there was no significant difference anymore between mature heads of warm- and cool-cultivated plants (Fig. 3 and Table 1) may indicate an acclimation of the all the time cool-cultivated plants to the lower temperature. In these plants the light-harvesting chlorophyll antenna may have been down-scaled and the chlorophyll a/b ratio altered (Havaux & Kloppstech, 2001). Thereby, again, the amount of energy captured and funnelled into the electron transport chain would be reduced and no anthocyanin accumulation would be necessary to encounter an enhanced oxidative load.

3.3.2. Flavonol and flavone glycosides

Regarding quercetin-3-*O*-(6″-*O*-malonyl)-glucoside, quercetin-3-*O*-glucuronide/luteolin-7-*O*-glucuronide, and quercetin-3-*O*-glucoside concentration, there were no significant differences between small heads that were cultivated either cool or warm (Fig. 3 and Table 1). Furthermore, there were no significant differences concerning these compounds between mature heads cultivated in different temperature regimes (Fig. 3 and Table 1).

If we compare warm- and cool-cultivated plants after the same number of days, we detect significantly higher concentrations of quercetin-3-*O*-(6″-*O*-malonyl)-glucoside and quercetin-3-*O*-glucuronide/luteolin-7-*O*-glucuronide (Table 2 and Fig. 3). However, the data of Romani et al. (2002) suggest a higher concentration of quercetin glycosides in early growth stage-lettuce compared to later stages. In Section 3.2 we demonstrated that warm- and cool-cultivated plants in our experiment were in different growth stages after 26 days of treatment. Hence, we conclude that the higher concentrations in the cool-cultivated plants were rather due to their growth stages than to the temperature treatment. This is in line with results Løvdal et al. (2010) obtained on leaves of tomato plants (*Solanum lycopersicum*): Quercetin glycosides were accumulated in response to increasing light intensity and nitrogen depletion rather than to lowered temperature alone. Indeed, quercetin glycoside concentration in red leaf lettuce does respond sensitively to radiation intensity (Becker et al., 2013). In our experiment, we closely monitored the macro nutrients in the nutrient solution to ensure they are sufficient and the PPFD we applied was constant (247 μmol m^{-2} s^{-1}).

The lowest temperature in our experiment (7 °C) was applied outside of the photoperiod and it, therefore, did not concur with radiation. Only with radiation present the photosystems can be over-excited and lead to extensive ROS production, i.e. photo-oxidative stress (Havaux & Kloppstech, 2001). Consistently, Boo et al. (2011) found higher anthocyanin concentrations in lettuce when low temperature was applied during the photoperiod than during the night. This interacting, enhancing effect of low temperature and radiation has also been reported for *Arabidopsis thaliana*, emphasizing that the combination of chilling and elevated PPFD is especially likely to induce photoinhibition and photo-oxidation in higher plants (Havaux & Kloppstech, 2001).

This may explain why our results differ from those of Oh et al. (2009). Apart from the different time span investigated (1 day as compared to several weeks in our experiment), they subjected their lettuce plants to 4 °C concurrent with radiation. Furthermore, they reduced the temperature by 16 K to 4 °C while we only reduced by 8 K to 7 °C. The larger magnitude of change and the application of a lower temperature during the photoperiod may exert more severe stress on plants and thus lead to an enhanced response.

The conditions we applied are more realistic regarding lettuce production in greenhouses than the drastic conditions applied by other studies.

In agreement with Løvdal et al. (2010), we conclude that in our experiment, the cyanidin glycoside truly responded to changes in temperature alone while quercetin and luteolin glycosides did not. As mentioned above (Section 3.3.1), an over-excited electron transport chain in chloroplasts mainly produces $O_2^{\cdot-}$ by electron transfer. Although cyanidin and quercetin are both flavonoids and both comprise an *ortho* 3′,4′-dihydroxy moiety, cyanidin has a higher $O_2^{\cdot-}$ scavenging activity than quercetin (Chun, Kim, & Lee, 2003). Quercetin on the other hand, is very effective against singlet oxygen ($^1O_2$) which is formed by energy transfer from excited triplet-state chlorophyll (Tournaire et al., 1993). The life time of triplet chlorophyll increases in excess radiation (Havaux & Kloppstech, 2001). This may explain the differential regulation of these two substances. This interpretation is corroborated by Gill and Tuteja (2010) who report that $^1O_2$ is involved in the activation of early stress response genes that are different from those activated by $O_2^{\cdot-}$.

3.4. Influence of temperature on phenolic acids

Cool-cultivated small heads contained higher concentrations of caffeoylmalic acid than warm-cultivated ones (Fig. 4 and Table 1). However, regarding mature heads, this difference is not detectable any more (Fig. 4 and Table 1). This also supports the hypothesis that the applied conditions were more stressful to small heads than to large**r** ones (see Section 3.3.1). Neither with small heads nor with mature heads we detected significantly different concen-

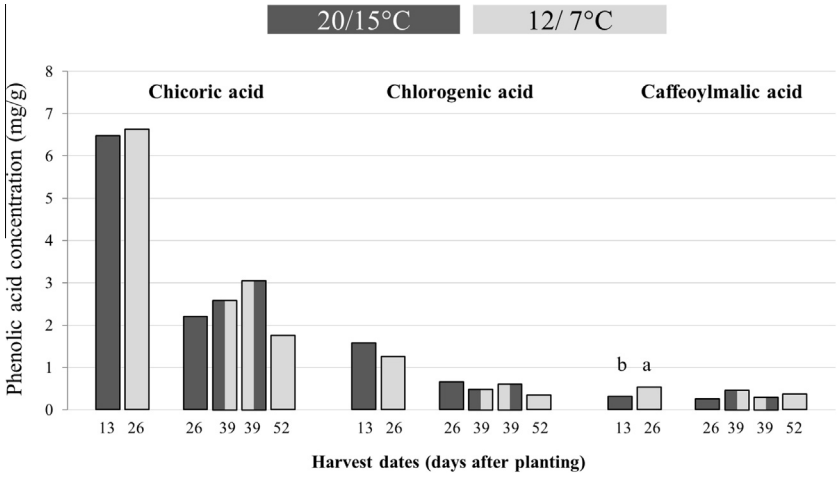

Fig. 4. Concentration of phenolic acids related to dry matter (DM) of red leaf lettuce, cultivated in different temperature regimes for a different number of days. For each compound, the first two bars represent small lettuce heads (days 13 and 26), while the other four bars (days 26, 39, 39, and 52) represent mature heads. For detailed description of the treatments, please see caption of Fig. 1. Identical letters on top of bars show that these treatments do not differ significantly ($n = 2$; Tukey-test, $\alpha = 0.05$).

trations of chicoric acid or chlorogenic acid between the temperature treatments (Fig. 4 and Table 1). Twenty-six days after planting, cool-cultivated plants contained higher concentrations of chicoric and caffeoylmalic acid than warm-cultivated ones, but this could not be detected for chlorogenic acid (Table 2). As we elucidate in Section 3.2, the plants compared were in very different growth stages and previously published results suggest that lettuce plants have higher concentrations of caffeoyl derivatives in early than in later growth stages (Romani et al., 2002). Hence, we do not suppose that the elevated concentrations can be interpreted as the plants' response to low temperatures but rather interpret this as a developmental bias.

Of the three phenolic acids that were evaluated, only the concentration of caffeoylmalic acid differed between plants cultivated in different temperature regimes, and only regarding small heads. This heterogeneity is in agreement with previously published results, indicating differences amongst phenolic acids regarding their response to environmental impacts (Oh et al., 2009) and amongst results obtained by different studies (Grace et al., 1998; Løvdal et al., 2010; Zidorn, 2010). Caffeoylmalic acid does not comprise the highest number of antioxidant structures per molecule (only one *ortho* 3′,4′-dihydroxy moiety whereas chicoric acid comprises one in each of the two caffeic acid moieties). Thus, we suppose the accumulation of caffeoylmalic acid in small heads has a function different from the commonly described antioxidant. Furthermore, there is no special similarity structure-wise between caffeoylmalic acid and cyanidin-3-*O*-(6″-*O*-malonyl)-glucoside which could explain why these two phenolic compounds were present in higher concentration in cool- than in warm-cultivated small heads. Unlike anthocyanins, phenolic acids do not absorb radiation in the wavelengths relevant for photosynthesis. Phenolic acids generally have their absorption maximum in the UV waveband and are therefore often considered UV protectants. However it is not very likely that UV played a role in our experiment as the applied radiation contained hardly UV radiation (HPS lamps; about 0.7% UV A and 0% UV B).

Løvdal et al. (2010) detected the strongest accumulation of caffeoyl derivatives in tomato leaves in response to a combination of high light, low nitrogen supply and low temperatures, indicating that temperature alone is not the trigger. Hence, the low-key impact we detected in our experiment might be due to our constant PPFD, the close monitoring of nutrient solution, and application of the lowest temperature outside the photoperiod.

4. Summary and outlook

We were able to confirm the hypothesis that low temperatures increase the concentration of flavonoids and phenolic acids in lettuce only for cyanidin-3-*O*-(6″-*O*-malonyl)-glucoside and caffeoylmalic acid: Their concentration was higher in cool-cultivated than in warm-cultivated small heads. This immediately leads us to the second and third hypotheses which could be fully confirmed: Dependent on their structure, different phenolic compounds vary in their response to low temperatures and the response in small heads is stronger than the one in mature lettuce plants. We confirmed two previous findings concerning growth and phenolic status of lettuce: Slower development with lower temperatures and higher concentrations of five out of seven studied phenolic compounds in smaller compared to larger plants.

The context of this experiment was to develop strategies to save energy during lettuce production in greenhouses in cool seasons, hopefully coinciding with higher concentrations of health promoting phenolic compounds. Unfortunately, these expectations have to be extenuated: When cultivated until large lettuce heads are formed, the concentration of phenolics in cool-cultivated plants will probably not be higher compared to warm-cultivated lettuce. However, especially in cool seasons, lettuce can be sold in earlier growth stages (100–150 g FM). These plants would not need as much time for cultivation, more plants could be grown per square meter (which are important economic aspects for producers) and they are, furthermore, very likely to contain higher concentrations of phenolic compounds than large heads. However, this has to be validated by greenhouse experiments under production conditions.

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