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Effects of photoperiod, growth temperature and cold acclimatisation on glucosinolates, sugars and fatty acids in kale

Anne Linn Hykkerud Steindal [a,*], Rolf Rødven [a], Espen Hansen [b], Jørgen Mølmann [a]

[a] Norwegian Institute for Agricultural and Environmental Research, Bioforsk Nord Holt, Box 2284, NO-9269 Tromsø, Norway
[b] Marbio, UiT The Arctic University of Norway, NO-9037 Tromsø, Norway

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ABSTRACT

Curly kale is a robust, cold tolerant plant with a high content of health-promoting compounds, grown at a range of latitudes. To assess the effects of temperature, photoperiod and cold acclimatisation on levels of glucosinolates, fatty acids and soluble sugars in kale, an experiment was set up under controlled conditions. Treatments consisted of combinations of the temperatures 15/9 or 21/15 °C, and photoperiods of 12 or 24 h, followed by a cold acclimatisation period. Levels of glucosinolates and fatty acid types in leaves were affected by growth conditions and cold acclimatisation, being generally highest before acclimatisation. The effects of growth temperature and photoperiod on freezing tolerance were most pronounced in plants grown without cold acclimatisation. The results indicate that cold acclimatisation can increase the content of soluble sugar and can thereby improve the taste, whilst the content of unsaturated fatty and glucosinolates acids may decrease.

1. Introduction

Curly kale (*Brassica oleracea* L. var *acephala*) is a biannual leafy vegetable, of the Brassicaceae family, being grown globally along a wide range of latitudes. Kale plants are robust and can tolerate cold temperatures below freezing. Its foliage is rich in nutrients and bioactive compounds, such as vitamins, minerals, glucosinolates and phenolic compounds (Ayaz et al., 2006). Epidemiological studies have shown an inverse relationship between consumption of *Brassica* vegetables and development of cancer and cardiovascular diseases (Jahangir, Kim, Choi, & Verpoorte, 2009). Anti-cancer attributes have been linked to glucosinolates and their degradation products, in addition to other compounds (Traka & Mithen, 2008; Verkerk et al., 2009). Glucosinolates are characterised by a core structure consisting of a β-D-thioglucose group, a sulfonated oxime moiety, and a variable side chain derived either from an aliphatic, aromatic or indolic amino acid (Moreno, Carvajal, López-Berenguer, & García-Viguera, 2006).

Growth conditions, such as day length and temperature, vary greatly with changing latitudes. Due to a low temperature optimum, kale is often grown in the cool season (late-summer until December) at low latitudes and in the summer season at higher latitudes (Decoteau, 2000). In areas above the Arctic Circle in Fennoscandia (66°N) the growth season (May–September) is characterised by low temperatures (9–14 °C average in Tromsø, Norway 70°N) and photoperiods up to 24 h. However, these 24 h photoperiods still have distinct night periods, with reduced radiation and lower temperatures at night (Nilsen, 1985). Broad latitudinal growing ranges have been reported to result in significant variation in the nutritive value and content of health-promoting components in some berries and vegetables (Hårdh, Persson, & Ottosson, 1977; Zheng, Kallio, & Yang, 2009). Although similar studies have not been conducted on *Brassica* crops, the content of glucosinolates in *Brassica* vegetables is affected not only by genotype variation, but also by environmental growth conditions, such as temperature and light conditions (Charron, Saxton, & Sams, 2005; Farnham, Wilson, Stephenson, & Fahey, 2004; Vallejo, Tomás-Barberán, Benavente-García, & García-Viguera, 2003; Velasco, Cartea, González, Vilar, & Ordás, 2007). In a previous study on broccoli (*B. oleracea* var. *italica*) we found higher content of glucosinolates at 21/15 °C compared to 15/9 °C, and also contents of individual glucosinolates were affected by day length (Steindal, Mølmann, Bengtsson, & Johansen, 2013). Schonhof, Kläring, Krumbein, Claußen, and Schreiner (2007) reported that low temperatures increased the content of aliphatic glucosinolates in broccoli, whilst high temperatures increased the levels of indolic glucosinolates.

Kale is highly tolerant to frost and it is not unusual that these plants remain unharvested in the field late in the growth season,

* Corresponding author. Tel.: +47 93 287 005.
E-mail address: anne.linn.hykkerud.steindal@bioforsk.no (A.L.H. Steindal).

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exposed to low temperatures and frost. Low temperature activates cold acclimatisation processes in plants, involving many biochemical and physiological changes, leading to enhanced tolerance to freezing (Levitt, 1980). These processes include growth reduction, accumulation of osmolytes, modification of membrane fatty acid composition and alterations in gene expression (Heino & Palva, 2004). Changes in levels of soluble sugars are often correlated to freezing tolerance for various plants (Savitch, Harney, & Huner, 2000). Soluble sugars are key contributors to cold acclimatisation as low molecular weight osmolytes accumulates in cells of frost tolerant plants. The accumulation of soluble sugars may also have an effect on taste in kale by increasing sweetness and tenderness of the leaves (Decoteau, 2000). Exposure of kale to low temperatures increases the content of soluble sugars, and is reported to both reduce (Hagen, Borge, Solhaug, & Bengtsson, 2009) and increase (Neugart et al., 2012) content of health-promoting phenolic compounds. Shifts in membrane fatty acid composition are a way for plants to protect membrane stability, integrity and function by increasing the proportion of unsaturated fatty acids (Steponkus, 1984). These cold-enhanced polyunsaturated fatty acids and their enhanced intake for humans are also linked to a range of health-promoting benefits (de Lorgeril & Salen, 2012).

Even though kale is a frost tolerant plant, there is little research on how low temperatures inducing cold acclimatisation affect the nutritional and health-promoting quality. In this paper we hypothesised that growth conditions associated with latitudinal growth sites can affect the levels of both the primary metabolite, sugars and fatty acids, as well as the secondary plant metabolite, glucosinolates, before and after a cold acclimatisation period. The frost tolerance is also believed to be affected by the conditions the plants have grown under before a cold acclimatisation period, in addition to the cold acclimatisation treatment which is known to increase the frost tolerance. With the increased interest in content of compounds associated with human health, elucidating the effects of environmental growth conditions and cold acclimatisation is of interest to growers and consumers.

In the present study the aim was therefore to see how the levels of glucosinolates, fatty acids and soluble sugars in kale related to temperature, photoperiod during growth and subsequent cold acclimatisation.

2. Material and methods

2.1. Plant material and experimental set-up

Different climatic conditions were simulated in temperature (±0.5 °C) and humidity controlled (0.5 kPa ± 3%) chambers (Phytotron of the University of Tromsø, Norway). Diurnal temperature was used to reflect different mean day and night temperature, in combination with contrasting photoperiods reflecting high and low latitudes. Kale (*B. oleracea* L. var *acephala* 'Reflex') seeds were germinated in a dark chamber (21 °C under 24 h light) in a fertilised peat/perlite soil mixture (1:1 v/v) under plastic cover. After germination (4 days), the plastic cover was removed and temperature was lowered to 15 °C. Two weeks after germination the plants were transferred to 12 cm diameter pots with a mixture of sand/compost and perlite (4:1 v/v). One month after germination, the plants were transferred to 12 l plastic pots with 10 l 70% sand/compost mixture and 30% perlite (8.5 kg) and fertilised with 12 g of a mineral nutrient (Yara, Fullgjødsel® 11-5-18 (NPK)) in the upper 5 cm of the soil. In addition, the plants were re-fertilised with 3 g calcium nitrate (Yara, Bor-Kalksalpeter, 15.4% N) every 6 weeks from the start of treatment until cold acclimatisation.

The experimental treatments were divided into two main parts: a growth period and a subsequent cold acclimatisation period. The experiments were set up in a $2 \times 2 \times 2$ factorial design. Initially plants were exposed to four different treatments; high (21/15 °C, day/night) or low temperature (15/9 °C, day/night) in combination with long (24 h) or short photoperiods (12 h). After the growth period, half of the plants were harvested and the other plants were exposed to cold acclimatised treatment.

During the growth treatments, photosynthetic active radiation was given for 12 h with fluorescent light tubes 165–175 μmol $m^{-2}s^{-1}$ (Phillips TLD 58W/840; Eindhoven, The Netherlands). The long photoperiod treatments were extended to 24 h with low irradiance lamps for 12 h, 6–8 μmol $m^{-2}s^{-1}$ (Osram delux el longlife 2020, Munich, Germany). The 12 h low irradiance extensions were in combination with the reduced night temperature. Eleven weeks after the germination period, 50% of the plants were harvested and the remaining plants exposed to a cold acclimatised treatment. All treatments had similar plant height except low growth temperature/short day which were about ~30% lower. The remaining plants were kept for 2 weeks at 6 °C, then two weeks at 3 °C and finally at 0.5 °C for 1 week. All plants received 12 h of low intensity light (6–8 μmol $m^{-2}s^{-1}$). There was little or no plant growth during the cold acclimatisation.

2.2. Freeze test

The effects of growth temperature, photoperiod and cold acclimatisation on frost tolerance were assessed by electrolyte leakage from leaf discs of kale leaves. Discs (diameter 11 mm) were cut out between main veins of the leaf number eight from the top leaf (the second fully extended leaf). Sixteen leaf discs from each treatment were placed between sheets of moistened paper towels and packed in moist sand in 2 l plastic boxes. The boxes were placed at 0.5 °C overnight before being moved to a temperature-regulated freezer at −3 °C for 17 h. Temperature was lowered at a rate of 1 °C h from −3 to −10 °C and then 3 °C h until 17 °C, and the boxes were removed each second degree. The temperature in the boxes was recorded with a thermocouple probe. After freezing, plants were thawed overnight at 4 °C. Leaf discs were then removed from the sand, and four leaf discs were placed in 5 ml of distilled water for 1.5 h in tubes with a lid. Leaf discs were also placed at continuous 0.5 °C for comparison. The electrical conductivity of the extract was measured by a digital conductivity metre (712 Conductometer, Metrohm, Switzerland) at room temperature. Each treatment had four replicates at each freezing temperature.

2.3. Chemical analysis

The first full expanded leaf, leaf number seven from the top was harvested for chemical analysis. The main vein was removed before being flash frozen in liquid nitrogen and stored at −80 °C. The samples were freeze dried and ground to a fine powder using a steel ball mill (Retsch MM301, Retsch GmbH, Haan, Germany).

2.3.1. Extraction and determination of glucosinolates

Freeze dried plant material (40 mg) was extracted according to Mellon, Bennett, Holst, and Williamson (2002) with some modifications. The glucosinolates were quantified using 0.05 μmol/sample glucotropaeolin as internal standard (AppliChem (A5300.0020), Darmstadt, Germany) applied by an automated pipette (HandyStep electronic repeating pipette, Brandtech Scientific Inc., Essex, CT, USA). Seven biological replicates were extracted per treatment and every tenth sample was extracted in duplicate to verify reproducibility (within 95%). Glucosinolates were analysed in randomised order by UHPLC–HR-MS on a Waters Acquity UPLC (Milford, MA) coupled to Waters LCT-Premier time-of-flight MS with electrospray ionisation. The glucosinolates were separated on a Waters Acquity charged surface hybrid (CSH) C18 column (2.1×50 mm, 1.7 μm) using a gradient of 2–30%

acetonitrile in water (both containing 0.1% formic acid) over 4 min at a flow rate of 0.6 ml/min. The column was kept at 40 °C and 1.00 µl of the glucosinolate extracts was injected. The samples were analysed by negative electrospray ionisation and *m/z* data from 150 to 1000 were acquired at a scan time of 0.25 s. Capillary and cone voltages were set at 2.4 kV and 50 V, respectively, whilst source and desolvation temperatures were set to 120 and 300 °C, respectively. Nitrogen was used as desolvation gas at 450 l/min. The MS was tuned to a resolution of 10,000 (FWHM) and leucine-enkapheline was infused through the reference probe for internal calibration during data aqusition. The peak for each glucosinolate (accurate mass ±0.05 Da) was integrated and the endogenous amounts were calculated based on the response of the internal standard.

2.3.2. Extraction and determination of fatty acids

Freeze dried plant material (20 mg) were extracted in freshly made methanolic 1 M HCl according to Browse, McCourt, and Somerville (1986). Detected fatty acids were quantified using 10 µg/sample heptadecanoic acid as internal standard (Fluka 51610), applied by an automated pipette. Seven biological replicates were extracted per treatment and extracted twice for analysis. Fatty acids were separated by gas chromatography using an Agilent 6890 N gas chromatograph equipped with a 7683 B auto injector and a flame ionisation detector (FID) (Agilent Technologies Inc., Santa Clara, CA, USA). Helium was used as carrier gas through a Varian CP7419 capillary column (50 m × 250 µm × 0.25 µm nominal) (Varian Inc., Middelburg, The Netherlands). Injector and detector temperatures were 240 and 250 °C, respectively. A predefined temperature program was used to ensure the best possible separation of the fatty acids: 50 °C for two minutes, then an increase of 10 °C/min to 150 °C, followed by 2 °C/min to 205 °C and finally 15 °C/min until 255 °C and 255 °C for 10 min. The fatty acids were identified by comparison to the fatty acid standards 1895, 1893, 1891, PUFA No. 1 and PUFA No. 3 from Sigma (Sigma Chemicals Co., St. Louis, MO, USA) and fatty acid standard 68D from NuChek (NuChek Prep. Inc., Elysian, MN, USA).

2.3.3. Extraction and determination of soluble sugars

Powdered freeze dried plant material (100 mg) was suspended in distilled water (40 ml) for one hour at room temperature, then centrifuged and filtered. D-Glucose, D-fructose and sucrose were quantified by enzymatic analyses using a Boehringer Mannheim test kit (Cat. No. 10 716 260 035, R-Biopharm AG, Darmstadt, Germany).

2.3.4. Statistical analysis

The full models for ion leakage and compound content included the predictors: temperature, day length, and cold acclimatisation and their second- and third-order interactions. For statistical inferences, we employed model selection using Akaike's information criterion corrected for small sample sizes, (AICc), a likelihood-based measure of model plausibility that penalises models with a higher number of parameters. In cases with $\Delta AIC < 1$, the model with the fewer parameters was assumed better (Burnham & Anderson, 2002). For freeze tolerance, the response variable ion leakage is a proportion, hence a general linear model with normal distribution and a logit link was used. For other responses, a general linear model with normal distribution and identity link was assumed. Particular variables were considered significant if their 95% confidence interval did not overlap zero. Differences among levels of a variable were identified using the Tukey test at the 95% significance level. Data were log transformed when necessary to ensure homogeneity of the variance. Data presented here are back-transformed for clarity. The statistical package R (version 2.15.0, R Development Core Team 2010; R Foundation for Statistical Computing, Vienna, AT) was used for all statistical analyses with the MuMIn package for model selection.

3. Results

3.1. Glucosinolates

Eight glucosinolates were identified in the kale leaves, belonging to three different chemical classes (Tables 1 and 2): three aliphatic, four indolic and one aromatic glucosinolate. Glucoiberin and glucobrassicin were the predominant glucosinolates in all treatments, with glucobrassicin, glucoraphanin, sinigrin, neoglucobrassicin, 4-hydroxyglucobrassicin, gluconasturtiin and 4-methoxy-glucobrassicin in decreasing order of concentrations for most treatments.

Both growth temperature and photoperiod affected the content of seven individual glucosinolates before the cold acclimatisation period. The responses varied depending on glucosinolate type, and several different interactions between growth temperature and photoperiod were observed. Long day combined with high growth temperature (21/15 °C) resulted in a ~45% lower content of glucoiberin compared to the other treatments. Whilst for the other two aliphatic glucosinolates, glucoraphanin and sinigrin, an effect of photoperiod and growth temperature was observed. Here the contents were higher at 12 h compared to 24 h photoperiod and lower at (15/9 °C) versus high (21/15 °C) growth temperature. Gluconasturtiin, the only aromatic glucosinolate, was detected in low amounts; however the content was highly affected by an interaction between the growth factors. Low growth temperature combined with 12 h day length led to the highest content of gluconasturtiin, ~4-fold higher than the other treatments. The indolic glucobrassicin was of higher content under 12 h versus 24 h day length at low growth temperature (15/9 °C), whilst there were no differences between day lengths at high growth temperature (21/15 °C). 4-Methoxyglucobrassicin was of higher content under 24 h at low growth temperature (15/9 °C) compared to the other treatments. Whilst only an effect of day length was observed for neo-glucobrassicin, with higher contents under 12 h versus 24 h.

After the cold acclimatisation period there were significant reductions in total glucosinolate content in plants that were previously grown at 12 h photoperiod in combination with low growth temperature (15/9 °C). This was due to significant reductions in the content of all individual glucosinolates for this treatment (Tables 1 and 2). Cold acclimatisation of plants from both growth treatments, 12 h at high growth temperature (21/15 °C) and 24 h at low growth temperature (15/9 °C), produced no significant change in the total content of glucosinolates, whilst leaves from 24 h photoperiod and high growth temperature (21/15 °C) had a slightly higher content after cold acclimatisation (Table 2). High growth temperature in combination with 24 h day length had a 51% higher content of glucoiberin after cold acclimatisation. Also, the content of sinigrin was generally highest after cold acclimatisation, and high growth temperature combined with 24 h photoperiod had a 65% higher content of sinigrin after cold acclimatisation. After the cold acclimatisation period, the levels of sinigrin were highest when the plants were previously grown at high growth temperatures. The predominant indolic glucosinolate, glucobrassicin, was in addition to being reduced in plants from 12 h at low growth temperature, also reduced by cold acclimatisation in plants from 24 h at low growth temperature.

A large variation in glucosinolate levels was detected within the treatments for glucoiberin, glucoraphanin, glucobrassicin, 4-hydroxyglucobrassicin and neoglucobrassicin, where the growing temperature and cold acclimatisation treatments explained less than 50% of the variation in the glucosinolate content. For glu-

Table 1
Content of aliphatic and aromatic glucosinolates in kale in kale grown under four different day length and temperature conditions, without and with a subsequent cold acclimatisation period (μmol/g dm, 95% confidence interval in brackets).

Temp. (°C) (day/night)	Day length (h)	Acclimatisation	Sinigrin	Glucoiberin	Glucoraphanin	Gluconasturtiin
15/9	12	–	1.06 (1.01–1.11)	5.50 (5.19–5.80)	1.55 (1.43–1.67)	0.30 (0.27–0.32)
15/9	24	–	0.71 (0.53–0.90)	5.66 (4.40–6.92)	1.00 (0.62–1.39)	0.07 (0.05–0.09)
21/15	12	–	0.72 (0.52–0.93)	5.70 (3.66–7.71)	1.06 (0.45–1.66)	0.08 (0.10–0.14)
21/15	24	–	0.60 (0.50–0.71)	3.86 (2.99–4.73)	0.81 (0.58–1.04)	0.14 (0.12–0.15)
15/9	12	+	0.74 (0.66–0.82)	3.81 (3.17–4.46)	0.84 (0.60–1.09)	0.15 (0.12–0.17)
15/9	24	+	0.76 (0.57–0.95)	4.46 (3.79–5.12)	1.07 (0.83–1.31)	0.13 (0.10–0.15)
21/15	12	+	1.12 (0.85–1.40)	5.50 (4.49–6.50)	0.69 (0.52–0.95)	0.05 (0.04–0.06)
21/15	24	+	1.40 (1.15–1.64)	5.91 (5.26–6.55)	0.87 (0.61–1.12)	0.05 (0.03–0.07)

$n = 7$, (12 h-21/15 °C, $n = 6$).

Table 2
Content of indolic glucosinolates in kale grown under four different day length and temperature conditions, without and with a subsequent cold acclimatisation period (μmol/g dm, 95% confidence interval in brackets).

Temp. (°C) (day/night)	Day length (h)	Acclimatisation	GB	4-OH-GB	4-Me-GB	Neo-GB	Total GLS
15/9	12	–	7.25 (6.62–7.87)	0.59 (0.50–0.68)	0.11 (0.10–0.13)	0.86 (0.83–0.90)	16.9 (15.8–18.0)
15/9	24	–	5.40 (3.76–7.05)	0.42 (0.28–0.56)	0.21 (0.15–0.27)	0.40 (0.22–0.58)	13.8 (10.3–17.4)
21/15	12	–	6.12 (3.43–8.80)	0.49 (0.29–0.69)	0.09 (0.06–0.13)	0.62 (0.26–0.99)	14.8 (9.4–20.2)
21/15	24	–	5.96 (5.67–6.25)	0.49 (0.42–0.56)	0.12 (0.11–0.14)	0.58 (0.48–0.68)	12.4 (11.2–13.7)
15/9	12	+	3.20 (2.65–3.76)	0.29 (0.24–0.33)	0.08 (0.07–0.09)	0.25 (0.15–0.34)	9.22 (7.87–10.6)
15/9	24	+	3.57 (2.89–4.26)	0.31 (0.28–0.35)	0.10 (0.09–0.11)	0.24 (0.06–0.43)	10.5 (8.83–12.2)
21/15	12	+	5.12 (2.38–7.85)	0.42 (0.23–0.62)	0.08 (0.05–0.12)	0.74 (0.15–1.33)	13.7 (9.33–18.1)
21/15	24	+	5.61 (4.41–6.81)	0.45 (0.36–0.53)	0.17 (0.12–0.21)	0.52 (0.23–0.81)	14.9 (13.2–16.6)

GB, glucobrassicin; 4-OH-GB, 4-hydroxyglucobrassicin; 4-Me-GB, 4-methoxyglucobrassicin; Neo-GB, neoglucobrassicin; GLS, glucosinolate. $n = 7$, (12 h-21/15 °C, $n = 6$).

conasturtiin, sinigrin and 4-methoxyglucobrassicin, more of the variation was explained by growing temperatures and cold acclimatisation treatments (60–92%).

3.2. Fatty acids

The most abundant unsaturated fatty acid was α-linolenic acid (18:3), and the most abundant saturated fatty acids were palmitic acid (16:0) and behenic acid (22:0) (Table 3).

Growth temperature and photoperiod were found to affect the content of fatty acids before cold acclimatisation. For α-linolenic acid (18:3) and stearic acid (18:0), a day length × temperature interaction was observed. The content of α-linolenic acid (18:3) at low growth temperature/24 h day length was 13% lower compared to low growth temperature/12 h day length. Oleic acid (18:1) and behenic acid (22:0) had the highest level at short day. The highest content of stearic acid (18:0) was found at high growth temperature and 12 h day length and 15/9 °C and 24 h day length. For hexadecatrienoic acid (16:3) the content at high growth temperature was 17% higher with a long photoperiodic regime. A difference in ratio between unsaturated fatty acids vs. palmitic acid (16:0) + stearic acid (18:0) was evident in the growth treatments, by having the highest ratio at 21/15 °C-24 h and lowest at 15/9 °C-24 h day length.

The data clearly showed that the fatty acid contents were highly affected by the cold acclimatisation period. The total content of fatty acids in kale subjected to cold acclimatisation were lower (9–25%) after cold acclimatisation. Indeed the oleic acid (18:1) fatty acid content was reduced by up to 91% (12 h; 15/9 °C) after cold acclimatisation whilst the content of behenic acid (22:0) was uniformly higher (~40%) (Table 3). The ratio between unsaturated fatty acids vs. palmitic acid (16:0) + stearic acid (18:0) had a higher portion of unsaturated fatty acids after cold acclimatisation (Table 3) for all combinations of growth temperature and day length, except the high growth temperature/24 h photoperiod. The ratio between unsaturated fatty acids and all saturated fatty acids did not appear to be impacted after cold acclimatisation, possibly due to a higher content of behenic acid (22:0) after cold acclimatisation.

3.3. Soluble sugars

D-Glucose and D-fructose were the two most abundant soluble sugars in kale leaves (Table 4). Photoperiod conditions affected the content of both D-glucose and D-fructose in leaves harvested prior to cold acclimatisation (Table 4). A 24 h photoperiod led to 63% and 88% higher content at 21/15 °C of D-glucose and D-fructose, respectively, whilst this effect was not detected at the lower growth temperatures. High growth temperature combined with 12 h day length however, reduced the content of both D-glucose and D-fructose.

The results showed that without exception levels of soluble sugar in the leaf tissue increased in response to cold acclimatisation (Table 4). The average content before and after cold acclimatisation increased with a factor of 1.3, 2.1 and 3.2 for D-glucose, D-fructose and sucrose, respectively. After cold acclimatisation, no difference in the content of the soluble sugars between growth treatments was detected (Table 4).

3.4. Ion leakage

Plants harvested prior to cold acclimatisation, generally had a higher ion leakage compared to cold acclimatised plants (Fig. 1). Before cold acclimatisation a day length temperature interaction was observed. The low growth temperature treatment, 12 h day length had higher freezing tolerance compared to 24 h day. After cold acclimatisation treatment there was also a significant lower ion leakage in the low growth temperature treatments. As observed before cold acclimatisation a temperature × photoperiod effect was seen: low growth temperature in combination with short day regime resulted in even lower ion leakage compared to long day, without cold acclimatisation.

Table 3
Content and composition of fatty acid in kale grown under four different day length and temperature conditions, without and with a subsequent cold acclimatisation period (mg/g dw, 95% confidence interval in brackets).

Temp. (°C)	Day length (h)	Acclimatisation	16:0[b]	16:3	18:0	18:1	18:2	18:3	22:0	USFA vs. SFA	USFA vs. 16:0 + 18:0
15/9	12	−	7.49 (7.16–7.81)	5.85 (5.53–6.17)	0.61 (0.58–0.65)	1.26 (1.19–1.33)	8.53 (8.19–8.87)	23.98 (23.0–25.0)	5.98 (5.11–6.85)	2.8 a[a]	4.9 de
15/9	24	−	7.36 (6.94–7.78)	5.69 (4.94–6.70)	0.71 (0.67–0.76)	0.96 (0.58–1.34)	8.19 (7.83–8.55)	21.19 (19.4–23.0)	4.86 (4.53–5.18)	2.8 a	4.4 f
21/15	12	−	7.54 (7.25–7.83)	5.69 (4.74–6.63)	0.71 (0.67–0.74)	1.30 (0.87–1.74)	8.38 (8.14–8.61)	22.29 (21.2–23.4)	5.47 (4.05–6.89)	2.8 a	4.6 ef
21/15	24	−	7.02 (6.67–7.36)	6.67 (6.03–7.32)	0.57 (0.51–0.64)	0.86 (0.56–1.15)	8.46 (7.92–8.99)	23.74 (21.9–25.5)	4.90 (4.60–5.20)	3.2 a	5.2 bc
15/9	12	+	5.40 (5.20–5.60)	4.61 (4.16–5.06)	0.29 (0.27–0.31)	0.11 (0.10–0.12)	6.21 (5.89–6.52)	20.26 (19.3–21.2)	7.97 (6.89–9.04)	2.3 b	5.5 ab
15/9	24	+	5.26 (5.14–5.38)	4.38 (4.19–4.57)	0.29 (0.28–0.30)	0.13 (0.11–0.14)	6.53 (6.22–6.84)	19.94 (19.7–20.2)	8.21 (7.06–9.35)	2.3 b	5.6 a
21/15	12	+	5.61 (5.35–5.89)	4.50 (4.18–4.81)	0.38 (0.36–0.40)	0.18 (0.15–0.20)	6.91 (6.52–7.29)	19.21 (18.1–20.3)	7.89 (7.26–8.52)	2.2 b	5.1 cd
21/15	24	+	5.37 (5.10–5.64)	4.07 (3.65–4.49)	0.35 (0.33–0.37)	0.15 (0.14–0.17)	6.99 (6.56–7.42)	17.66 (16.2–19.1)	7.19 (6.28–8.11)	2.2 b	5.0 cd

SFA, saturated fatty acid; USFA, unsaturated fatty acid.
[a]Means with no common letter within columns are significantly different ($P \leqslant 0.05$), $n = 7$.
[b]Membrane fatty acids.

4. Discussion

In general, the highest content of glucosinolates in the present study was found in kale leaves prior to cold acclimatisation. Low growth temperatures accompanied by frost can result in degradation in leaves, which may reduce glucosinolate contents (Fenwick, Heaney, & Mullin, 1983). Velasco et al. (2007) studied the content of glucosinolates in kale grown in different field conditions, and reported that low growth temperature down to freezing temperatures led to lower glucosinolate content. Conversely, they also found that leaves harvested in the coldest month (January) had a slightly higher glucosinolate content. The dominating glucosinolate in these kale plants was sinigrin. An increase after low growth temperature exposure was also found for sinigrin in our study. We also found the variation of glucosinolates between individual plants within each treatment to be high. The variation in glucosinolate content has also previously been found to vary greatly between individual broccoli plants (Borowski, Szajdek, Borowska, Ciska, & Zieliński, 2008). Our results in combination with other studies suggest that cold acclimatisation temperatures can give a reduction in overall glucosinolate content; however this appears to depend on the type of glucosinolate and pre-acclimatisation growth conditions.

Growth conditions affected the content of some of the individual glucosinolates. Accumulation of glucosinolates has previously been reported to be affected by growth temperature and photoperiod in leaves of rapid-cycling *B. oleracea*, watercress (*Nasturium officinale* R. Br.) and in broccoli florets (Charron & Sams, 2004; Engelen-Eigles, Holden, Cohen, & Gardner, 2006; Steindal et al., 2013). It has generally been observed that high temperatures are favourable for high glucosinolate content, and indolic glucosinolates have been reported to respond most strongly to environmental factors in broccoli heads (Brown et al., 2002; Farnham et al., 2004). However, we did not find highest indolic contents at the higher growth temperatures. Indolic glucosinolates were less affected by the treatments compared to both aliphatic and aromatic glucosinolates. This is in agreement with a study on cabbage seedlings (*B. oleracea* var. *capitata*) where there was no correlation between growth temperature and level of glucosinolate (Rosa & Rodrigues, 1998). Likewise, aliphatic glucosinolates in broccoli were also highest at growth temperatures below 12 °C (Schonhof et al., 2007). Content of aliphatic and total glucosinolates in leaves were highest at high (32 °C) and low (12 °C) temperatures, whilst the indolic glucosinolates increased with increasing temperature in rapid cycling *B. oleracea* (Charron & Sams, 2004). Our results may indicate that low growth temperature stress can increase the content of glucosinolates; however, other studies (Brown et al., 2002; Charron & Sams, 2004; Engelen-Eigles et al., 2006; Farnham et al., 2004) have found that these kinds of effects depend highly on plant organs, species and also the range of the temperature being tested.

In addition to the effect of growth temperature, photoperiod also affected levels of some of the individual glucosinolates. This effect was not unidirectional, being both higher and lower at 12 h day length. Charron and Sams (2004) found no significant effect of photoperiod in leafs in rapid-cycling *B. oleracea*. However, in a previous study in broccoli we generally found a reduction of aliphatic glucosinolates due to 24 h photoperiod in combination high (21/15 °C) temperature (Steindal et al., 2013). This was also observed for the aliphatic glucosinolates, sinigrin and glucoraphanin, at 15/9 °C in the present study as well as for some of the indolic and the aromatic glucosinolate. A negative effect of a 24 h light period on glucosinolates may be linked to the lack of circadian rhythm, which is involved in the regulation of glucosinolates in *Arabidopsis* (Huseby et al., 2013). The effect of day length in our

Table 4
Content of soluble sugars in kale grown under four different day length and temperature conditions, without and with a subsequent cold acclimatisation period (g/100 g dw, 95% confidence interval in brackets.

Temp. (°C)	Day length (h)	Acclimatisation	D-Glucose	D-Fructose	Sucrose	Total
15/9	12	–	3.0 (2.9–3.2)	2.5 (2.2–2.7)	0.5 (0.3–0.6)	6.0 (5.6–6.4)
15/9	24	–	2.9 (2.5–3.3)	1.9 (1.7–2.1)	0.6 (0.4–0.8)	5.3 (4.7–6.0)
21/15	12	–	2.2 (1.5–2.9)	1.7 (1.5–1.9)	0.7 (0.2–1.1)	4.5 (3.6–5.4)
21/15	24	–	3.6 (3.2–4.1)	3.2 (2.7–3.7)	0.6 (0.2–1.1)	7.4 (6.1–8.7)
15/9	12	+	4.7 (4.4–5.0)	4.8 (4.6–5.1)	1.5 (1.2–1.8)	11.1 (10.5–11.7)
15/9	24	+	5.0 (4.3–5.6)	5.0 (4.1–5.2)	2.1 (1.4–3.2)	12.1 (9.9–13.7)
21/15	12	+	5.0 (4.8–5.3)	4.6 (4.5–5.5)	2.2 (1.7–2.4)	11.8 (11.3–12.9)
21/15	24	+	4.7 (4.5–4.8)	4.6 (4.3–4.8)	2.2 (1.5–2.9)	11.4 (10.8–12.1)

$n = 5$.

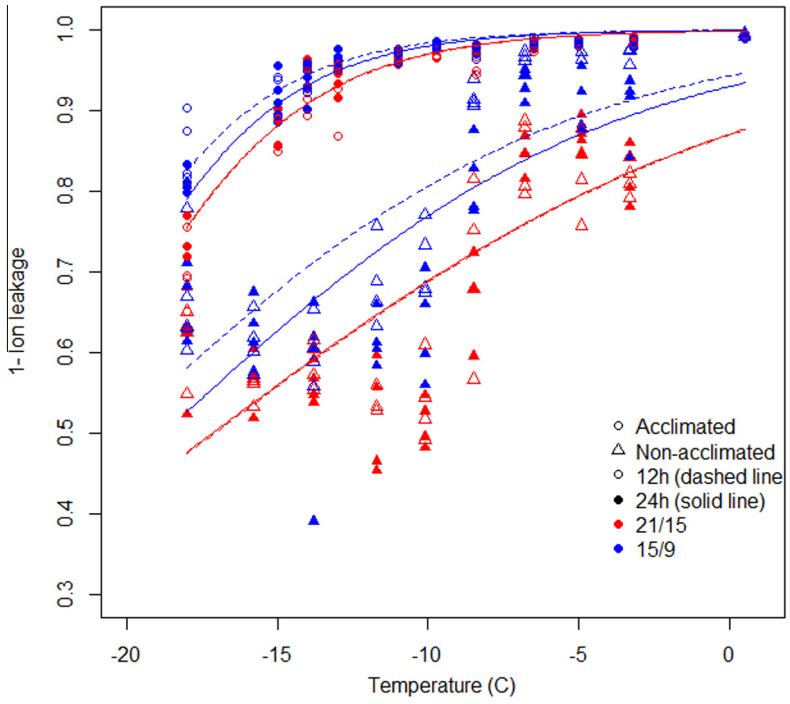

Fig. 1. Effects of pre-acclimatisation temperature and photoperiod and cold acclimatisation treatments on electrolyte leakage in kale leaf. The electrolyte leakage is presented as back transformed data of freezing tests from before and after cold acclimatisation period, and the lines of best fit were calculated using logit transformed data. The damage was estimated as percentage electrolyte leakage for samples held at each of the indicated temperatures. Triangles are not acclimatised, circles are acclimatised. Filled symbols/solid lines are 24 h photoperiod. Open symbols/dotted lines are 12 h photoperiod. Gray lines are high temperature, 21/15 °C, and black lines are low growth temperature, 15/9 °C.

study seems to be dependent upon growth temperature, since several of the effects of photoperiod were only found at either high or low growth temperature.

The electrolyte leakage measurements indicate a positive relationship between low growth temperature and short photoperiod in combination with cold acclimatisation periods on frost tolerance (Fig. 1). In a recent study of *Arabidopsis*, Lee and Thomashow (2012) reported that long, warm days were accompanied by a repression of activity in the C-repeat binding factor pathway, responsible for cold acclimatisation, and that repression is relieved as day length shortens, thereby increasing frost tolerance.

The content of fatty acids have, as in our study, previously been found to decrease with increasing temperature (20–22 and 12–17 °C) in rapeseed (*Brassica napus*), soybeans (*Glycine max* L.), sunflowers (*Helianthus annuus*) and cauliflower (*B. oleracea* var. *botrytis*) (Werteker, Lorenz, Johannes, Berghofer, & Findlay, 2010), and the ratio of unsaturated vs. saturated fatty acids was higher when grown in late compared to early seasons in Italy (Lo Scalzo, Bianchi, Genna, & Summa, 2007). The content of sucrose in two *Hydrangea* species increased under short day at low growth temperature conditions (Pagter, Liu, Jensen, & Petersen, 2008). These findings suggest that the content of both fatty acids and soluble sugars can to some extent be influenced by preharvest growth temperatures and photoperiod in plants.

An increase in both the content of soluble sugars and a higher ratio of unsaturated membrane fatty acids occurred in plants after cold acclimatisation periods in our study, and this was related to increased frost tolerance. The main observed change in fatty acid content in cold acclimatised kale leaves, was the overall reduction of the total unsaturated fatty acids. The highest frost tolerance among non-acclimatised plants was observed in plants grown at the lower temperatures (15/9 °C). This treatment did not have the highest content of either unsaturated fatty acids or soluble sugars, suggesting that frost tolerance in non-acclimatised plants is not directly linked to the level of soluble sugars and unsaturated fatty acids. However, the ratio of unsaturated versus membrane fatty acids palmitic acid (16:0) + stearic acid (18:0) increased after cold acclimatisation, which together with decreased ion leakage

indicates that the degree of unsaturated fatty acids may reflect the freezing tolerance. The increase of behenic acid (22:0) after cold acclimatisation may be due to behenic acid (22:0) being a constituent of epicuticular wax rather than a membrane fatty acid (Millar, Smith, & Kunst, 2000). A relative increase in its content in epicuticular wax was reported in *Arabidopsis* plants exposed to 11 days of cold acclimatisation at 4 °C (Amid, Lytovchenko, Fernie, & Thorlby, 2012). The higher ratio of unsaturated fatty acids in our study indicates that they are part of the cold acclimatisation process in kale, and that a degradation process can reduce the general content of membrane fatty acids as seen in the current study. It has been reported, both under natural and controlled conditions, that the content of soluble sugars increases when plants are subjected to low growth temperatures. The content of soluble sugars has previously been found to increase in curly kale when exposed to subzero temperatures (Hagen et al., 2009). Also in cabbage seedlings (*B. oleracea* L. cv. *Banchurisou*) an increase of the soluble sugars were detected during cold acclimatisation and caused increased frost tolerance (Sasaki, Ichimura, & Oda, 1996). Strand et al. (1999) demonstrated that up-regulation of sucrose synthesis during cold acclimatisation is essential for the development of freezing tolerance in *Arabidopsis*. In our study, the content of sucrose followed by D-fructose had the highest increase, whilst the increase of D-glucose content was only minor before and after cold acclimatisation. It is thus likely that both composition of unsaturated membrane fatty acids and higher levels of soluble sugars, especially sucrose and D-fructose, are linked to frost tolerance in kale.

5. Conclusion

In conclusion, the present study shows that cold acclimatisation conditions generally led to some reductions in the content of both total and individual glucosinolates. Although, some glucosinolates were unaffected by cold acclimatisation and sinigrin was higher. In addition, the pre-cold acclimatisation temperature and photoperiod conditions also affected the content, short day and high temperatures being favourable for the main aliphatic glucosinolate, glucoiberin. The overall fatty acid contents were lower after cold acclimatisation and the highest level of α-linolenic acid (18:3) was observed at 12 h day length at low growth temperatures. The content of soluble sugars, especially sucrose and D-fructose was highest after exposure to cold acclimatisation. This means that, to optimise high-intake of unsaturated fatty acids and glucosinolates from kale, it may be more favourable to harvest leaves before exposure to low acclimatisation temperatures, in the late autumn/winter season. Sugars on the other hand would increase the sweetness of the leaf when exposed to low acclimatisation temperatures. These findings mean that careful and precise harvest planning can balance sensory quality and health beneficial properties.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.10.129.

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