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A Cold Case—Glucosinolate Levels in Kale Cultivars Are Differently Influenced by Cold Temperatures

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Abstract: Among the *Brassica oleracea* L. crops, kale has gained increased global recognition in recent years as a healthy food item due to its high nutritional value and versatility. Additionally, the diversity of different kale varieties has started to be explored across large latitudes from the Mediterranean to north temperate climates. Specifically, glucosinolates are the predominant phytochemicals found in kale leaves, contributing to the specific taste of this vegetable, and they are affected by environmental factors such as temperature. To date, no study has investigated the effect of chilling on glucosinolate diversity and, thus, the taste in genetically different kale cultivars at the same time. Given the variability of glucosinolates observed among cultivars, we evaluated the impact of acclimation to cold temperatures on glucosinolate levels in curly kale, Lacinato kale, and a feral type using high-performance liquid chromatography coupled with time-of-flight mass spectrometry (HPLC-ESI-qTOF-MS). We targeted the short-term impact (after 12 h) on glucosinolates as well as the longer-term effect (after seven days) of cold acclimation. Our results revealed different molecular patterns regarding the change in glucosinolates in the feral type compared to curly kale and Lacinato-type kale. In the latter ones, primary aliphatic glucosinolates were induced (the glucoraphanin in Lacinato kale increased by more than 200%). The indole glucobrassicin was not significantly affected. Conversely, in the feral type the indole glucobrassicin was reduced by 35% after cold acclimation, whereas aliphatic glucosinolates were hardly affected. The results indicate that both genetic and environmental factors are important for the composition of glucosinolate patterns in kale. In conclusion, to obtain plants with an improved nutritional value, considering both temperature and the choice of cultivar is crucial during kale cultivation. Future breeding attempts of kale should also emphasize the cultivar-dependent cold acclimation patterns reported here.

Keywords: *Brassica oleracea*; Lacinato; glucosinolates; HPLC-ESI-MS; chilling; cold acclimation

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1. Introduction

The availability of a nutrient-rich diet throughout the whole year is important for well-balanced human nutrition. Nowadays, this is assured through frozen food, but in the past, few vegetables were available as fresh food in wintertime. Among the vegetables that are freshly harvested regularly during the winter season, such as field salad, allium, parsnip, endive, or Brussels sprouts, kale (*Brassica oleracea* var. *sabellica* L.) has been referred to in recent years as a superfood [1]. However, the difference in nutritional value between harvest in summer, as commercially performed nowadays, and in winter after longer cold acclimation has hardly been investigated. Kale is valued for its high amounts of manifold beneficial metabolites (including vitamins, mineral nutrients, carotenoids, polyphenols, and glucosinolates) and for the versatility of its preparation as part of the diet. Apart from Northern Germany where kale has been well established for hundreds of years, kale is gaining popularity globally in recent times and is recognized as an everyday food item.

The general versatility of kale is paralleled by an enormous range of plants denominated as "kale", comprising more than 120 different varieties and cultivars, of which some are only locally known and which occur from the Mediterranean climate to a north temperate climate. This diversity can be grouped as either "curly kale", "Lacinato kale", "collards", "Russian/Siberian kale", or "feral types", as demonstrated by Hahn et al. [2]. They differ not only genetically, but also in terms of growth, leaf morphology, color, phytochemicals, and taste [3]. Feral-type cabbages are particularly interesting since they are likely to have originated from plants that escaped from domestication and now grow mostly as hardy, big-leaved herbs on European coastal limestone cliffs [4].

Given the huge diversity of varieties, a broad range of different tastes can be found in kale, ranging from bitter to nutty to mild and sweet. The taste of kale is determined by both low-molecular-weight carbohydrates (LMWC) [5], phenolic compounds [6], and glucosinolate-derived volatiles [7]. The latter ones, glucosinolates, are the major secondary plant metabolites in *Brassica* crops and, thus, in kale [8]. They are derived from amino acids and play an important role in the chemical defense mechanism of the plants, as well as being beneficial to human health due to the chemopreventive and anti-inflammatory effects of their breakdown products [9–12]. Most of them are associated with a bitter and pungent taste (cf. Table 1).

All glucosinolates have a core structure in common, consisting of a β-D-glucopyranose (six-membered D-glucose ring) connected via a sulfur atom to a (*Z*)-*N*-hydroximinosulfate ester ($NOSO_3^-$), as well as a variable side chain (R-group) determining the specific characteristics of each glucosinolate (cf. Table 1). The biosynthesis of glucosinolates is controlled by a couple of quantitative trait loci (QTLs) [13] and regulated—among other factors—by brassinosteroid plant hormones, as suggested by Lee et al. [14].

More than 130 glucosinolates are known to date (Blažević et al. [15] and Nguyen et al. [16] give a recent thorough overview on these), of which about 20 have been identified in *Brassica* species [17]. In parallel to the large genetic and morphological diversity of kale, our previous analysis of glucosinolate composition in 25 kale varieties at harvest demonstrated a notable variation among varieties and cultivars of different origins [18] with specific patterns for curly kale, Lacinato kale, and feral types, respectively. Glucosinolates can be classified threefold according to the amino acid they have been derived from: aliphatic (derived from methionine, alanine, valine, leucine, or isoleucine), aromatic (tyrosine or phenylalanine), or indole glucosinolates derived from tryptophane [19] (cf. Table 1).

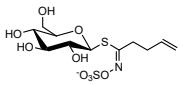
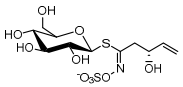
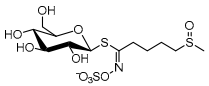
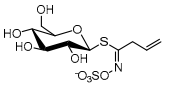
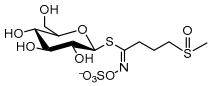
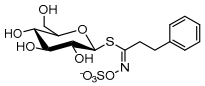
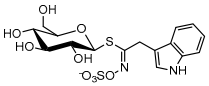
Glucosinolates are located within the cell vacuole. Upon plant damage, they are hydrolyzed to glucose, sulphate, and one of various products, such as isothiocyanates, thiocyanates, indoles, nitriles, or epithionitriles [20], by an enzyme (myrosinase)-catalyzed Lossen rearrangement. The main products derived from the glucosinolates commonly found in *Brassica* species are summarized in Table 1.

For the plants, the glucosinolate–myrosinase system plays a role in the alleviation of biotic as well as abiotic stresses. Glucosinolates and their metabolites (especially isothiocyanates) mainly function in the growth inhibition and feeding deterrence of a wide range of herbivores and pathogens, such as insects and slugs [21–23]. Besides herbivory, glucosinolates also play a role in the plant's defense against various abiotic stresses, such as light, temperature, or drought, as reviewed by Variyar et al. [24], among others. Especially for *Brassica*, the effect of environmental stresses on glucosinolates has been reported in prior studies [25,26]. In response to environmental stressors, signal transduction pathways are activated that alter the levels of specific glucosinolates [24]. Studies have suggested that indolic glucosinolates are more easily induced by such stresses than aliphatics (e.g., Kushad et al. [27]). Furthermore, the considerable variation of glucosinolates has been observed not only among tissue and plant age [28,29], but also among cultivars within a species [30,31], with regard to both concentration and composition.

Undoubtedly, among these environmental influences, temperature is the most important factor for plants grown during fall and winter, such as kale. Jurkow et al. [32]

demonstrated in their study that cold stress increases the level of biologically active ingredients in curly kale. A recent study has shown that chilling temperatures affect plant compounds such as LMWC in kale cultivars [33]. Regarding glucosinolates, Cartea et al. [34] and Steindal et al. [35] found lower glucosinolate levels in kale plants in winter and after five weeks of cold acclimation, respectively. In contrast, Ljubej et al. [36,37] reported that specific glucosinolates gained a higher concentration in kale at chilling temperatures, which is in line with the report of Kissen et al. [31] for *Arabidopsis*. In any case, the reported results reveal that the concentrations of glucosinolates in kale plants are definitely affected by cold temperatures and presumably are involved in the cold stress tolerance of kale. However, to what extent glucosinolates play a role in plant protection against freezing is not fully understood yet, despite their importance for taste and potential health benefits.

Table 1. The seven glucosinolates analyzed in this study, with their relevant characteristics, their main breakdown products (bioactive metabolites), the corresponding sensory attributes [7,12,38], and their main activities after human consumption. Chemical structures have been drawn using ChemDraw software (v. 15.0.0.106, PerkinElmer Informatics, Boston, MA, USA). Molecular formulas were obtained from The Royal Society of Chemistry [39]. ITC = isothiocyanate, TC = thiocyanate.

Group	Trivial Name	Abbreviation	Structure	Molecular Formula	*m*/*z* [M-H]		Error (ppm)	Retention Time (min)	Post-Hydrolysis Derivatives (Bioactive Metabolites)	Sensory Descriptor	Main Activities in Conjunction with Human Consumption
					Exptl	Theor					
aliphatic	gluconapin	GN		$C_{11}H_{19}N_1O_9S_2$	372.0399	372.0418	4.9	7.0	3-butenyl ITC	bitter taste, pungent	induction of cancer-cell apoptosis [40]
	progoitrin	PR		$C_{11}H_{19}N_1O_{10}S_2$	388.0368	388.0366	2.5	4.4	goitrin (oxazolidine-2-thione)	bitter taste	taste [41], possibly causing goiter [42]
	glucoraphanin	GR		$C_{12}H_{23}N_1O_{10}S_3$	436.0396	436.0400	3.5	4.3	sulforaphane (ITC)	no taste	anti-infective [43], antiviral [43], antagonizing angiogenesis and tumor-cell metastasis [44], apoptosis induction [45]
	sinigrin	SIN		$C_{10}H_{17}N_1O_9S_2$	358.0339	358.0344	2.1	5.7	allyl ITC (AITC), allyl TC	bitter taste, pungent	odor-active [41], taste [41], several therapeutic benefits [46]
	glucoiberin	GI		$C_{11}H_{21}N_1O_{10}S_3$	422.0325	422.0327	1.9	9.1	iberin (ITC)	no taste	anticarcinogenic [47]
aromatic	gluco-nasturtiin	GA		$C_{15}H_{21}N_1O_9S_2$	422.0578	422.0574	1.7	17.0	2-phenylethyl ITC (PEITC)	pungent	anticarcinogenic [48], antibacterial [48]
indole	glucobrassicin	GB		$C_{16}H_{20}N_2O_9S_2$	447.0496	447.0537	2.6	15.8	indole-3-carbinol (I3C), 3,3-diindolylmethane (DIM)	bitter taste	high scavenging activity [49]

Nevertheless, research assessing different kale varieties is rare, especially regarding the effect of cold temperatures on phytochemical levels in kale. In the recent studies of Ljubej and colleagues, the authors evaluated the effect of chilling and freezing temperatures on glucosinolate levels in kale plants during short-term exposure (24 h) [36] and long-term exposure (7 days) [37], as well as the effect of 24 h low temperatures on kale sprouts [50]. In all of their experiments, chilling temperatures induced an increase in glucosinolates. However, the authors chose only one local Croatian non-curly cultivar, as described in an earlier publication [51]. Although the results provide valuable information about cold-stress-related glucosinolate changes in kale, it is not clear whether this is true for other kale varieties as well. To date, we are not aware of any study that has specifically evaluated the influence of cold-temperature stress on glucosinolate levels of a set of several different kale varieties. Furthermore, it is worth noting that in Northern Germany, non-commercial kale harvest is traditionally recommended following the first frost, supposedly increasing kale sensory quality [3,52].

To the best of our knowledge, the present study is the first that investigated the effect of chilling on glucosinolate diversity in genetically different kale cultivars. Given the wide range of variability of glucosinolates and the cultivar-dependent patterns previously reported for kale [18], the objective of our study was, therefore, to quantitatively investigate the impact of acclimation to cold temperatures on the composition and level of glucosinolates in three kale cultivars from different origins (curly type, Lacinato type, and feral type). We targeted the short-term impact on glucosinolates as well as the long-term effect after seven days of cold acclimation. We expected, based on previous data, the levels of sinigrin, glucoiberin, progoitrin, glucoraphanin, and gluconasturtiin to be raised as a result of cold-temperature exposure [35,37,53], and the level of glucobrassicin to be decreased [35]. Furthermore, we expected Mediterranean Lacinato kale to react differently to cold temperatures than Northern German curly and feral types based on different climatic adaptations. Our present study provides the necessary foundation for transcriptomic studies on the effect of low temperatures on the phytochemistry of *Brassica oleracea* and its changes within the plants.

2. Materials and Methods

2.1. Plant Material

Representatives of the three main kale groups [2] were chosen, i.e., curly-type kale "Frostara" (obtained from Bruno Nebelung GmbH, Everswinkel, Germany), Lacinato-type kale "Palmizio" (obtained from Thompson & Morgan Ltd., Ipswich, UK), and wild cabbage "Helgoländer" (feral type, seeds harvested at the Botanical Garden Oldenburg) as representative for collard kale types (Figure 1). 45 individuals of each cultivarwere seeded in multipot trays (substrate Hawita-Flor P + Ton 4007, HAWITA Gruppe GmbH, Vechta, Germany) and cultivated in a climate chamber (const. 25 °C, 60–70% air humidity, 4000 lux light intensity, light/dark 12 h/12 h) (Tecto Standard Plus, Viessmann Kühlsysteme GmbH, Hof, Germany) at the Botanical Garden Oldenburg. When the plants reached the 5-leaf stage, they were transplanted into single pots (3 L) with potting soil containing clay, perlite, calcium carbonate, and Osmocote and Radigen fertilizers. They were watered and fertilized (Wuxal Super, Aglukon, Düsseldorf, Germany) according to standard cultural practices. Voucher specimens of the kale varieties used are deposited at the herbarium of the Carl von Ossietzky-University, Oldenburg (OLD).

Figure 1. Kale and cabbage varieties chosen for this study: (**A**) Curly kale "Frostara". (**B**) Lacinato-type kale "Palmizio". (**C**) Feral-type cabbage "Helgoländer".

2.2. Climate Chamber Experiment

After 6 weeks, fresh leaf material was collected from 15 individual plants of each variety (referred to as "warm" sampling, i.e., day 0 before cold acclimation). We collected 4–5 of the upper fully developed leaves, packed them in paper bags, and brought them to the laboratory. Immediately afterwards, the temperature inside the climate chamber was changed to 2 °C and the remaining plants were left at this temperature for seven days. Further leaf samples were taken after 12 h and 7 days of cold exposure, respectively ("12 h cold" and "7d cold" sampling). At each time-point, 15 individual plants per variety were randomly chosen, harvested as described above, and removed from the climate chamber. The experiment was repeated for control plants that were constantly grown at 25 °C (meaning no temperature change), leaving all other conditions the same. Leaf samples of these control plants were taken at the very same time-points. The choice of the time-points was based on prior studies on glucosinolate accumulation in *Brassica* species [37,54].

2.3. Extraction of Glucosinolates from Plant Material

The extraction method was described previously in Hahn et al. [18]. Briefly, 10 g of each freshly collected sample (see above) was weighedin (by mixing material from all leaves of the particular sample and excluding thick midnerves) and dried at 120 °C for 2 h. The dried leaves were subsequently ground to a fine powder, each sample mixed with 8 mL of 70% *v*/*v* methanol/water (of HPLC grade; Fisher Scientific, Loughborough, UK), and sonicated for 15 min. The resulting supernatant was filtered (0.45 µm pore size) and stored afterwards at −20 °C until further analysis.

2.4. Quantification of Seven Glucosinolates with HPLC-ESI-qTOF-MS

Seven glucosinolates were targeted in this study, i.e., glucoiberin (hereafter abbreviated as GI), sinigrin (SIN), glucoraphanin (GR), gluconapin (GN), progoitrin (PR), gluconasturtiin (GA), and glucobrassicin (GB) (Table 1). The choice of these glucosinolates was based on prior studies reported in the literature to cover glucosinolates from which major bioactive metabolites are derived, and to ensure that all chemical glucosinolates groups were met.

After the thermal deactivation of the enzyme myrosinase, kale leaf extracts were profiled as aqueous methanolic extracts using an adapted liquid chromatography mass spectrometry method (LC-MS) [55,56]. In detail, an HPLC-qTOF-MS coupling with negative ion electrospray ionization (ESI) was used for the high sensitive detection and quantification of the glucosinolates. The same Bruker impact HD quadrupole time-of-flight (qTOF) mass spectrometer (Bruker Daltonics GmbH & Co. KG, Bremen, Germany) coupled to an Agilent 1260 Series HPLC system (Agilent Technologies, Santa Clara, CA, USA) was used as described in Hahn et al. [18], with the same parameters specified there, concerning column, mobile phase, and MS-scan parameters. Before analysis, 2 mL of the extracted samples were filtered (0.45 µm pore size) into 2 mL screw top vials and then loaded into

the HPLC. Eluate absorbance was monitored at 229 nm UV. MS-scans were performed in negative ion mode on the glucosinolate $[M\text{-}H]^-$ ions. MS-spectra were recorded between *m/z* (mass-to-charge ratio) 100 and 700. Quantification was carried out on extracted ion chromatograms of each respective glucosinolate *m/z* value with an isolation width of 0.002 Da. Six-point calibration curves (Supplementary Information Figure S1) were obtained from seven analytical standard glucosinolates (PhytoLab GmbH & Co. KG, Vestenbergsgreuth, Germany) as reference for the quantification of the extracted samples (cf. Table 2). The Pearson correlation coefficient R^2 was calculated for each calibration curve. The lowest quantifiable amount for each glucosinolate (limit of quantification, LOQ) was set to a concentration yielding a signal-to-noise ratio (S/N) of 10:1 (Table 2). Mass calibration was performed by injecting a 0.01 mM sodium formate solution prior to each chromatographic run using an enhanced quadratic calibration algorithm. Kale extracts spiked with authentic reference compounds showed a relative standard deviation (RSD) below 5%, indicating that no appreciable matrix effect was in operation.

Table 2. Parameters for quantification of glucosinolates: Slope, axis intercept, and Pearson correlation coefficient R^2 of calibration curves, as well as relative standard deviation (RSD) of analytical repeat injections and LOQ values as the lowest quantifiable amount (S/N ratio of 10:1).

Glucosinolate		Slope	Axis Intercept	R^2	RSD (%)	LOQ (mg/L)
GN	gluconapin	0.0036	2.7525	0.9963	3.97	3.49
PR	progoitrin	0.0048	0.5658	0.9997	3.01	1.33
GR	glucoraphanin	0.0074	0.2895	0.9996	4.16	1.25
SIN	sinigrin	0.0046	0.3270	0.9989	3.84	1.25
GI	glucoiberin	0.0034	0.2931	0.9993	3.91	0.99
GA	gluconasturtiin	0.0030	−1.0210	0.9975	4.37	0.01
GB	glucobrassicin	0.0068	−0.5779	0.9986	3.23	0.17

2.5. Data Analysis

Data were evaluated using ESI Compass 1.3 Data Analysis software (v. 4.2.383.1, Bruker Daltonics GmbH & Co. KG, Bremen, Germany). Calculation of glucosinolate contents was completed using Microsoft Excel (v. 16.0, 2016, Microsoft Corporation, Washington, DC, USA). Results were expressed as mg 100 g^{-1} fresh weight (FW). Statistical analyses were performed with the software R (v. 4.2.0, R Core Team [57]) (File S1 in Supplementary Information).

3. Results

Seven glucosinolates were quantified via HPLC-ESI-TOF-MS in three different kale types (curly type, Lacinato type, and feral type) grown in warm temperature conditions (25 °C) and after cold acclimation (2 °C). The main analytical parameters are summarized in Table 2. Calibration curves obtained from analytical reference substances were found to have correlation coefficients R^2 of >0.99. Chromatographic reproducibility was confirmed with repeat injections yielding a relative standard deviation (RSD) <5%. LOQ values are indicated for an S/N ratio of 10:1. The assignment of glucosinolates was carried out through a comparison of retention time, high-resolution *m/z* value, and MS^2 spectra to authentic standards. The glucosinolates were identified in the chromatogram by the high-resolution *m/z* value of their [M–H] pseudomolecular ion, which all showed errors below 5 ppm. Isothiocyanate products (such as sulforaphane) were not present in the LC-MS chromatograms.

Changes of Glucosinolate Patterns during Cold Acclimation

The resulting glucosinolate values determined in kale plants before and after cold acclimation are presented in Table 3, with 15 individual plants per variety measured at each time-point. The glucosinolate values of all individual samples can be found in Tables S1–S3 of the Supplementary Information.

Besides large variation in biological replicates that were also observed in the main experiment (detailed in the discussion), there were no significant changes in the glucosinolate levels of the control plants between the sampling points (Table 3 and Supplementary Information Figure S3). The glucosinolate values of all individual control samples can be found in Tables S4–S6 of the Supplementary Information.

The aromatic glucosinolate GA as well as the aliphatic GI were not detected in any of the three varieties and at any sampling time-point.

In the main experiment, the highest glucosinolate levels were found in the leaves of the feral type, reaching up to 192.3 mg 100 g^{-1} FW for GB (Figure 2). For the aliphatic glucosinolates (SIN, GR, GN, and PR), we found quantities between 0.52 mg 100 g^{-1} FW (GN at warm temperatures) and 70.9 mg 100 g^{-1} FW (GR after 7 days cold acclimation). For SIN, GN, and PR, there were no major differences between warm (25 °C) and cold (2 °C) temperature conditions ($p > 0.6$ each) (cf. also Figure S2A,C,D in the Supplementary Information). The GR values, however, were observed to be slightly raised as a result of cold temperature application (Figure S2B), although not significantly ($p = 0.41$). In contrast, though also not significant ($p = 0.18$), quantities of the indole glucosinolate GB decreased after the temperature changed from warm to cold (Figure S2E). This decline continued the longer the plants were exposed to the cool temperature. As mentioned, GB was the glucosinolate with the highest amounts found in the feral type, both at warm and cold temperature conditions (min. 11.5—max. 192.3 mg 100 g^{-1} FW).

In general, except for GB, the amount of glucosinolates detected in curly kale "Frostara" was quite low (Figure 2). For both aliphatics SIN and PR, values between 0.4 and 1.4 mg 100 g^{-1} FW were detected, with the highest values found after 7 days cold acclimation (Figure S2A,D; cf. Table S1 in the Supplementary Information). Clear differences between the treatments were not visible ($p = 0.32$ and 0.45, respectively). For GR (Figure S2B), equally small amounts were found (0.5–2.0 mg 100 g^{-1} FW). However, the amount of GR after 7 days of cold acclimation was significantly higher compared to warm temperature conditions ($p = 0.01$). The predominant glucosinolate in "Frostara" kale was, as in the feral type, GB (Figure S2E), with values ranging from 6.1 to 69.4 mg 100 g^{-1} FW. The highest amounts were found after 12 h of cold exposure, though no significant differences between warm and cold conditions could be spotted ($p = 0.42$). GN could not be detected in any of the "Frostara" samples, neither at warm conditions nor after cold acclimation.

In the Lacinato kale variety "Black Tuscany", only two glucosinolates were found, namely GR and GB (Figure 2). The GR level ranged from 0.7 to 19.5 mg 100 g^{-1} FW (Figure S2B), with significantly higher amounts present after 7 days cold treatment versus the 12 h treatment ($p = 0.02$), as well as compared to warm conditions ($p = 0.02$). The amount of GB varied greatly between the analyzed samples (between 2.7 and 139.2 mg 100 g^{-1} FW; Figure S2E) but did not differ between warm and cold temperature conditions ($p = 0.18$).

Table 3. Glucosinolate levels (mg 100 g^{-1} FW) of kale cultivar "Frostara" (F), kale cultivar "Black Tuscany" (B), and feral-type "Helgoländer" (W), determined in plants cultivated in warm temperature conditions ("warm") as well as after cold acclimation ("12 h cold" and "7d cold", respectively). Data shown as mean and the minimum and maximum values (in brackets). nd = not detected. Different letters in the same cultivar at different time-points indicate significant differences ($p < 0.05$). For abbreviations of glucosinolate names, refer to Table 1.

Cultivar	Treatment	Sampling	Glucosinolates (mg 100 g^{-1} FW)						
			GN	PR	GR	SIN	GI	GA	GB
F	cold	warm (day 0)	nd	0.83 (0.83)	0.65 (0.48–1.02) [a]	0.63 (0.48–0.81)	nd	nd	30.30 (6.12–57.52)
		12 h	nd	0.47 (0.45–0.49)	0.85 (0.57–1.18) [ab]	0.56 (0.44–0.68)	nd	nd	37.68 (12.23–69.37)
		7d	nd	0.77 (0.40–1.44)	1.21 (0.80–2.00) [bc]	0.85 (0.52–1.41)	nd	nd	31.26 (18.75–56.93)
	control (warm)	day 0	nd	nd	nd	nd	nd	nd	40.74 (19.55–67.94)
		12 h	nd	nd	nd	nd	nd	nd	48.39 (12.77–104.51)
		7d	nd	nd	nd	nd	nd	nd	41.33 (19.30–92.03)
B	cold	warm (day 0)	nd	nd	1.82 (0.66–5.93) [a]	nd	nd	nd	53.37 (2.69–139.16)
		12 h	nd	nd	1.83 (0.91–3.95) [a]	nd	nd	nd	34.57 (4.21–73.69)
		7d	nd	nd	6.10 (0.70–19.45) [b]	nd	nd	nd	61.18 (7.12–130.56)
	control (warm)	day 0	nd	nd	nd	nd	nd	nd	41.01 (5.92–93.91)
		12 h	nd	nd	nd	nd	nd	nd	50.63 (22.05–126.03)
		7d	nd	nd	nd	nd	nd	nd	67.42 (35.45–119.66)
W	cold	warm (day 0)	10.50 (0.52–34.75)	17.57 (2.27–40.19)	4.20 (0.59–12.96)	4.63 (0.84–12.15)	nd	nd	90.11 (11.50–192.31)
		12 h	7.90 (0.84–20.37)	20.78 (6.73–46.59)	15.46 (0.77–51.55)	3.39 (3.39)	nd	nd	86.83 (18.91–182.14)
		7d	8.23 (0.53–30.79)	17.37 (6.99–34.00)	16.32 (0.82–70.92)	3.82 (0.61–7.02)	nd	nd	60.81 (24.77–150.33)
	control (warm)	day 0	1.04 (0.45–3.97)	6.19 (0.51–21.31)	4.53 (0.40–11.98)	3.58 (0.63–7.17)	nd	nd	150.67 (17.44–356.33)
		12 h	0.80 (0.39–1.28)	5.48 (0.87–20.93)	3.50 (0.45–6.55)	3.39 (1.47–6.70)	nd	nd	149.69 (26.84–329.89)
		7d	1.16 (0.44–1.75)	6.73 (1.23–19.83)	5.84 (0.56–9.20)	2.86 (0.55–7.80)	nd	nd	125.79 (16.53–268.24)

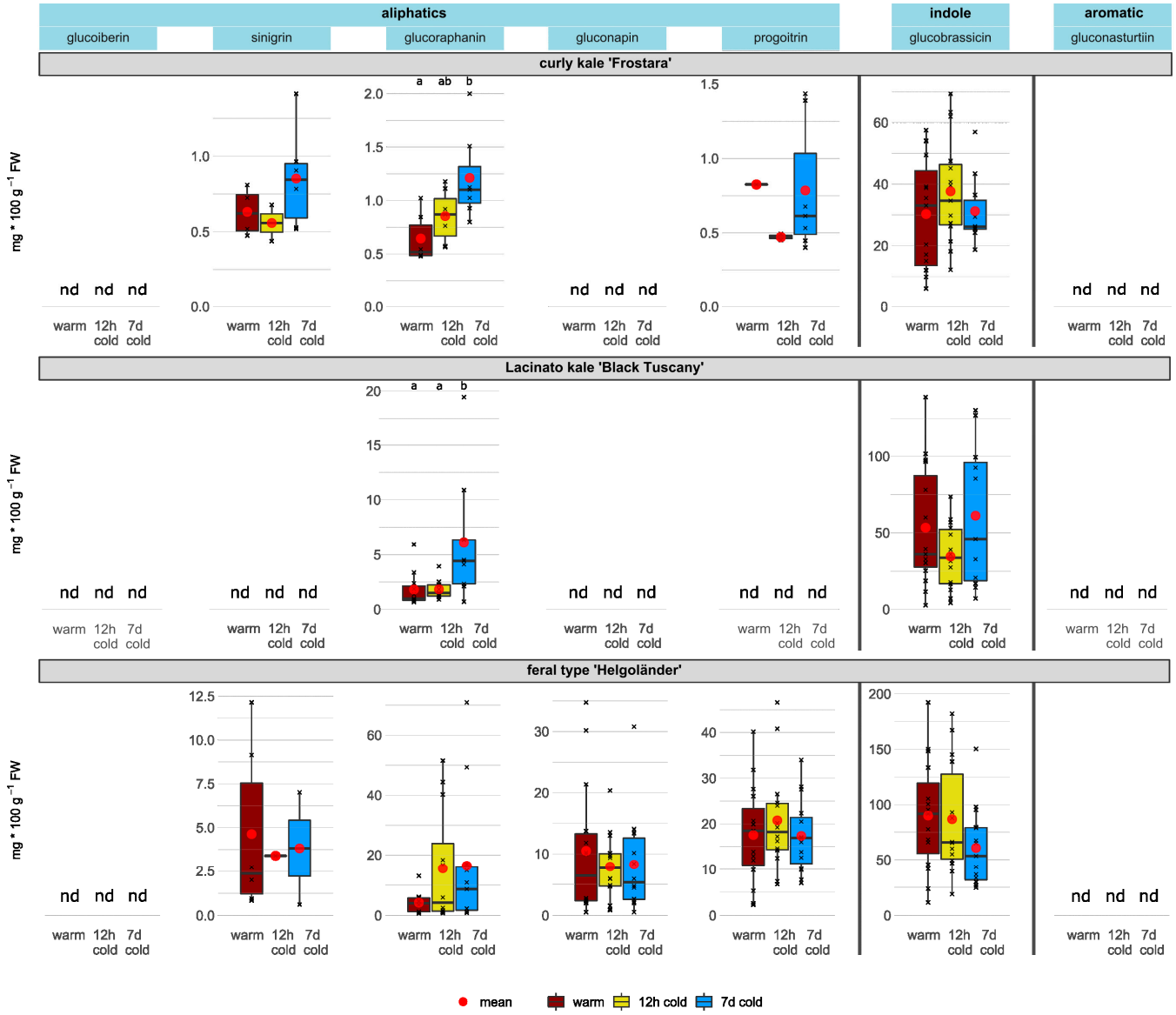


Figure 2. Glucosinolate levels (mg 100 g^{-1} FW) in different kale cultivars (curly kale "Frostara", Lacinato kale "Black Tuscany", and feral-type "Helgoländer") cultivated in warm temperature conditions ("warm") as well as after cold acclimation ("12 h cold" and "7 d cold", respectively). Individual data points (crosses) as well as mean and median are shown. Note the different scales. nd = not detected. Different letters above bars indicate significant differences at $p < 0.05$.

4. Discussion

4.1. Chilling from the Kale's Perspective—Change of Glucosinolate Pattern in Cold-Treated Kale

Evaluating the influence of cold temperatures on glucosinolate levels in the leaves of different kale cultivars was made possible by quantifying seven major glucosinolates via HPLC-ESI-MS. Based on the results, curly kale "Frostara" reacts to cold-temperature exposure with an induction of GR production by approx. 85% (Figure S2B). The longer the plants were exposed to chilling, significantly more GR was present in the leaves (although on a relatively low scale). A similar pattern was observed for PR and SIN (Figure S2A,D), but due to a small sample size, this pattern was not significant. GB—being already present in substantial amounts at warm temperatures—was, however, slightly induced by approx. 25% within 12 h cold treatment, followed by a subsequent reduction of 17% (Figure S2E). Concerning GN, this aliphatic glucosinolate was completely absent in curly kale, which is in line with the report of Nilsson et al. [30] for a Swedish curly

kale variety and for other kale varieties studied by Hahn et al. [18]. It seems that, for curly kale "Frostara", the aliphatic glucosinolates reacted faster to temperature stress than the indole GB. The recent investigation of low-molecular-weight carbohydrates in kale has shown that sugars, including glucose, become available at low temperatures as well [33]. Assuming that glucosinolate metabolism is linked to carbohydrate metabolism, the increase in glucosinolates as a result of cold temperatures may, therefore, be considered to be a serendipitous side effect: The large amount of glucose available as a result of low temperatures is used to build more glucosinolates.

Similar to the pattern reported for curly kale "Frostara", Lacinato kale "Black Tuscany" also reacts to chilling temperatures by inducing GR production (by approx. 235%) but more slowly (Figure S2B). For GB, in contrast, the levels are slightly (and non-significantly) reduced within the first 12 h of chilling by approx. 35%. This is the opposite pattern to kale "Frostara", in which GB is rather induced within this period. Since GB is clearly more abundant in Lacinato kales than GR, it is, however, apparent that there is a strong diversion of precursors to the indolic branch of glucosinolate production rather than for the aliphatic, which is in line with the report of Ferioli et al. [58] for Lacinato kales.

The reaction of the feral type to the chilling environment is different compared to the other two varieties. First, although we observed a slight increase in GR production (Figure 2), this pattern was not significant and not as clear as in kales "Frostara" and "Black Tuscany". There were huge differences in GR levels among the feral-type samples, but many samples did not differ notably between the three treatments (warm, 12 h cold, 7 days cold) suggesting that GR is present constitutively. Second, we observed a visible (though non-significant) trend to lower GB values in feral types the longer the plants were exposed to chilling by approx. 35% (Figure 2). Several explanations may be possible for the decrease in GB glucosinolates with low temperatures. It can be supposed that either the GB synthesis is down-regulated as a result of cold stress, or the plants switch to the metabolization of GB to other indolic glucosinolates (such as neoglucobrassicin or 4-hydroxyglucobrassicin [59]), which we did not investigate since we wanted to restrict the group of indolic glucosinolates to the parent compound indolylmethyl glucosinolate (GB). Such a switch would be supported by results that abiotic stress affects the chain elongation step of glucosinolates biosynthesis [26]. A third alternative would be that GB molecules were broken down to release the glucose moiety that subsequently served as a substrate for the membrane-protecting carbohydrate compounds involved in the protection of photosynthetic infrastructure against damage from cold stress [33,60]. Shattuck et al. [61] reported cold-temperature-induced tissue disruption and, thus, the degradation of glucosinolates upon contact with myrosinase, although glucosinolates may be released from the vacuole into the cytosol even without tissue disruption upon stress [26]. Lastly, glucosinolates may have been translocated to other parts of the plants, as suggested by Rosa et al. [62]. Still, the relevance of glucosinolates for freezing protection in plants remains largely unclear. Besides the abundance of GB, a raised importance of the aliphatic branch of glucosinolate biosynthesis in the feral-type cabbage is generally obvious, specifically emphasizing the conversion of GR to GN and further side-chain modifications to PR. However, during cold stress, indole glucosinolates are apparently of greater importance for the feral type than aliphatics (since GN, PR, and SIN were not affected by cold temperatures here), which contrasts with the patterns of "Frostara" and "Black Tuscany" kales described above.

The different glucosinolate patterns found in the different kale types are indicators of the genetically different kale cultivars. Overall, the highest glucosinolate values in total were found in the feral-type plants, both in warm and cold conditions ($p < 0.01$). Surprisingly, curly kale "Frostara" was the one with the lowest values in total, much lower than expected. In the study of Hahn et al. [18], "Frostara" was, for example, among the kale varieties with the highest GR levels of more than 40 mg 100 g^{-1} FW. The discrepancy may likely be due to the fact that, in our experiment, we did not deal with field-grown plants but with climate chamber conditions, for which such differences have been reported before [63]. Apparently, our intention of subjecting the plants to cold-temperature stress

did work, especially with regard to the fact that after 7 days of cold acclimation, a couple of plants had been observed as becoming droopy and soft. However, these plants had to be sorted out due to an insufficient amount of fresh leaf material for the sampling. Concerning the Lacinato kale "Black Tuscany", the present study could largely confirm previous findings [18], which expect the plants to generally contain rather low glucosinolate levels, except for GR and GB.

Glucosinolates are protective substances against herbivores. In late autumn, there are still herbivores such as slugs around that can pose a threat to the kale plants. Although they would hide when it becomes really cold, there are still other large herbivores, such as pigeons, for which kale offers a freshly available food source in winter when little else is available. The observed increased production of glucosinolates in response to low temperatures can be seen as a strategy of the plants to be prepared against the feeding of such herbivores apart from its involvement in countering abiotic stress, even in winter.

4.2. Chilling from the Glucosinolates' Perspective

With regard to the synthesis of aliphatic glucosinolates, the biosynthetic branch that leads to the synthesis of GI and SIN from a methylthioalkyl glucosinolate precursor seems to be much less used in kale than the parallel branch, resulting in GR, GN, or PR [64]. It seems that the latter (i.e., the GR biosynthetic pathway) is key in kale cultivars exposed to cold stress. In our experiment, GR levels were raised in all three cultivars the longer they had been exposed to cold temperatures (in the feral type at least slightly). The enzymes that catalyze the conversion of GR into GN (controlled by an *ALK* locus) and GN into PR (*OH* locus) [65] must be more highly expressed in feral types and less expressed in curly kale and Lacinato kale. However, to date there is no evidence for this in the literature, and specific transcriptome analyses comparing different kale cultivars are needed here.

In accordance with our previous findings [18], GR considerably varied between different kale plants and cultivars. In curly kale, GR was significantly induced in cold temperatures. Since GR, after enzymatic degradation, leads to beneficial bioactive metabolites such as sulforaphane (one of the most potent compounds targeting multiple actions within the cell) [66], an elevated GR level in kale plants is of great value and is crucial to consider in future breeding attempts of kale.

For SIN, we observed a reaction to the temperature treatment only in curly kale "Frostara" (where SIN increased, though not significantly). This is in accordance with the observation of Steindal et al. [35], who reported SIN in curly kale "Reflex" to be the highest after cold acclimation. In contrast, in the feral-type SIN was not affected in our analysis but was 4–6 times higher initially compared to "Frostara". Given the fact that the breakdown products of SIN are highly odor-active compounds and contribute to the perception of bitterness and hotness [67,68], a high level of SIN may be selected for in feral types as an effective herbivore deterrent and should be avoided in cultivated kale plants to prevent consumer rejection.

GN has only been found in the feral type but did not show a significant reaction to the cold treatment. Low levels or the absence of GN in curly and Lacinato types has previously been found [18] and the presence of GN found here in the feral type and in American collard kales by Hahn et al. [18] suggests a closer phytochemical relationship of these plants both characterized by flat, leathery leaves.

A low or even undetectable PR value (as in curly and Lacinato kale) is more preferable since the breakdown products of PR (i.e., goitrin) have undesirable properties [42]. However, there seems to be a low risk of developing thyroid diseases for humans regularly consuming *Brassica* vegetables as recent studies suggest [69,70]. The feral type turned out to be much worse in this regard with PR being the second most abundant glucosinolate, again in agreement with results for American collards [18]. This suggests, supported by phylogenetic relationships [2], that curly kale was actively bred for low SIN and PR levels from ancestral collard types with higher PR (and possibly SIN) levels [18].

For the indole glucosinolate GB, comparing the three kale cultivars revealed a remarkable pattern. In the feral type, the amounts in warm temperature conditions as well as after 12 h of cold acclimation were clearly higher than in the other two kales ($p < 0.001$). However, with ongoing chilling the GB levels in all three cultivars approached each other and became not significantly different after 7 days ($p > 0.1$). A high GB amount in kale is beneficial given the health protective characteristics of its post-hydrolysis derivatives indol-3-carbinol (I3C) and 3,3-diindolylmethane (DIM) [49]. However, its effect on plants in chilling conditions has not been studied.

GA and GI were found in all samples to be below the detection limit and, thus, could not be quantified. Considering the findings of Charron et al. [71], GA is neither present in kale in spring nor in fall. It is likely that the biosynthetic pathway for aromatic glucosinolates is disrupted or down-regulated in kale. Concerning GI, although it has not been found in any of the samples in our analysis, it is likely that most GI has been converted to its derived propenyl-glucosinolate SIN in these plants.

Humans are very sensitive to even low concentrations of glucosinolates [38]. Despite the various health benefits reported for its consumption, attempts to enrich glucosinolates in vegetables for health may result in rejected consumer acceptance. Thus, competing demands of taste and health are an important aspect for the food industry [38], as well as for the future breeding of kale types.

4.3. Within-Cultivar Variation and the Issue of Low Glucosinolate Values

For most glucosinolates, we found rather low values and, in many plants, a couple of glucosinolates could not be detected at all. It is likely that the amounts of these respective glucosinolates were so low in the leaves that, with our LC-MS method, it was not possible to detect them (they were below the detection limit). In consequence, this results in a reduced sample size for some of the treatments and, thus, lowers the credibility of putative trends observed in the data (i.e., the differences between warm and cold temperature conditions). Furthermore, for most of the glucosinolates, we observed a huge variation among samples of the same variety. This was true for all treatments, regardless of warm or cold temperatures. This large variation in biological replicates was also observed for kale in previous studies [18,33,72,73] and can be explained by the intrinsic differences of the individual plants. To better deal with variation in glucosinolate concentrations, the sample size may be enlarged in future studies.

Additionally, the growth chamber itself, or rather, the long timespan over which the plants were grown may have been problematic and not ideal for kale plants. The tendency of a couple of plants was to appear to be unnaturally slender and soft; the Lacinato kale "Black Tuscany" in particular did not perform well. Poorter et al. [63] pointed out that phenotypic differences in plants from growth chambers are common. Although in our study, all the important growth parameters were set up sufficiently in the chamber (temperature, light, water, nutrient supply, and fan), the plants may still have suffered from lacking a natural environment. Specifically, the lack of proper wind may have led to the reduced stability of the plants [63,74]. Concerning the constant growth temperature during the development of the plants, it may have been too high during the first weeks of the experiment for the development of naturally robust seedlings. However, since our aim was to assess the impact of temperature change on the glucosinolate levels, we decided on a constant warm temperature from the beginning. Thus, it appears that glucosinolate values were either heterogeneous between plants or completely below the detection limit. This is especially true for the control samples. Here, in kales "Black Tuscany" as well as "Frostara", only GB could be detected, and nearly a third of the Lacinato kale plants had to be sorted out due to insufficient quality, leading to a sample size $n < 15$.

5. Conclusions

In this study, we targeted the short-term impact as well as the long-term effect of cold acclimation on the glucosinolate composition in kale. Curly and Lacinato-type kales ("Frostara" and "Black Tuscany", respectively) both reacted to cold acclimation primarily by inducing aliphatic glucosinolates (such as GR, GN, PR, and SIN) by more than 200% compared to warm conditions. The indolic GB, however, was not affected in the long term. The main reaction of the feral type was, in contrast, a reduction of GB content after cold acclimation by 35%. The aliphatics were not affected in this cultivar, despite a slight increase in GR. Thus, here, we observed the activation of different molecular processes affecting the biosynthesis of glucosinolates in the feral type during cold acclimation compared to the other cultivars. Differently than expected, despite generally lower levels or even the complete absence of glucosinolates in the Mediterranean Lacinato kale, this cultivar did not react notably differently to cold acclimation compared to curly kale. Our results demonstrate that genetic factors are at least equally as important for the composition of glucosinolates in kale as environmental factors. We recommend that commercial kale breeders and home gardeners consider both temperature and cultivar specificity when breeding kale for improving the nutritional value. Further determination of specific glucosinolate profiles for certain groups of kale cultivars under cold or other stresses is, therefore, beneficial in terms of breeding kale types for improved nutrition. The results obtained in the present study present important foundations for transcriptomic studies on the effect of low temperatures on *Brassica oleracea's* phytochemistry.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9090953/s1, Figure S1: Calibration curves of the seven glucosinolates included in this study; Figure S2: Overview of glucosinolate levels in the three different kale cultivars cultivated in warm temperature conditions as well as after cold acclimation; Figure S3: Glucosinolate levels of control plants cultivated at constant warm temperatures; Tables S1–S6: Glucosinolate levels of all individual plants measured; File S1: R-script of the analyses carried out (R-file).

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Abbreviations

ESI, electrospray ionization; FW, fresh weight; GA, gluconasturtiin; GB, glucobrassicin; GI, glucoiberin; GN, gluconapin; GR, glucoraphanin; HPLC, high performance liquid chromatography; LMWC, low molecular weight carbohydrates; LOQ, limit of quantification; *m*/*z*, mass-to-charge ratio; MS, mass spectrometry; PR, progoitrin; qTOF, quadrupole time-of-flight; S/N, signal-to-noise ratio; SIN, sinigrin.

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