Journal of Plant Physiology 317 (2026) 154695

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Screening frost-tolerant kale (*Brassica oleracea* L. var. *acephala)* genotypes through cold-responsive metabolic changes in open field conditions

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ARTICLE INFO

Keywords:
Leafy kale
Glucosinolate
Sugars
Pigments
Frost stress
Metabolic plasticity

ABSTRACT

Kale (*Brassica oleracea* L. var. *acephala)* is a cold-tolerant leafy vegetable whose metabolic plasticity under frost stress remains underexplored. In this study, leaf tissues from 26 kale accessions grown under open-field conditions were metabolically profiled, focusing on soluble sugars, glucosinolates, and photosynthetic pigments before and after exposure to short-term frost. Frost stress induced significant quantitative and compositional shifts in sugar profiles, notably an accumulation of sucrose, suggesting its role as a key osmoprotectant. Among the twelve glucosinolates identified, indolic compounds dominated the unfrosted profile (67.4 %) but declined post-frost (51.6 %), coinciding with a marked increase in aliphatic glucosinolates (from 20.7 % to 38.6 %). Chlorophyll and carotenoid contents declined in most accessions following frost exposure. Notably, the metabolic profile of Accession_4 indicates a potentially resilient phenotype, characterised by limited pigment degradation and a shift toward aliphatic glucosinolates. This may reflect a stress-adaptation strategy and could be explored as a candidate for breeding or metabolotype selection approaches.

1. Introduction

Among different varieties of *Brassica oleracea,* kale has gained importance as a nutritious food and is attracting increasing attention worldwide due to its remarkable nutritional profile and culinary versatility. Due to its high content of vitamins, minerals and bioactive compounds, kale has become a favoured choice for a health-oriented diet (Becerra-Moreno et al., 2013; Giorgetti et al., 2017). With the increasing popularity of kale, research has expanded to explore the various kale varieties found in a diverse range of climates, from the Mediterranean to the northern temperate regions (Ferioli et al., 2013). In recent decades, the morphological, agronomic, genetic and phytochemical characteristics of local kale varieties have been extensively studied in numerous countries (Balkaya and Yanmaz, 2005; Cartea et al., 2003; Šutković et al., 2021). The research emphasises not only the adaptability of kale to different environmental conditions but also its potential as a resilient crop. Leafy kale is one of the most cold-tolerant plants in the Brassicaceae family, thriving at temperatures just a few degrees above freezing and can withstand extremes down to −20 °C (Hahn et al., 2023; Ljubej et al., 2021). Despite its resilience, cold stress can affect the growth and productivity of kale, potentially leading to significant yield losses. Low temperatures affect many physiological and biochemical responses in plants, mainly by promoting the production of reactive oxygen species (ROS). These ROS can alter the composition of membrane fatty acids and trigger changes in gene expression, which in turn stimulate the production of radical scavengers and osmoprotectants to maintain cellular homeostasis and protect against oxidative damage (Sanghera et al., 2011).

Plant responses to abiotic stress are highly complex and involve coordinated regulation at multiple levels, including gene expression, hormonal signalling antioxidant defence and osmoprotectant accumulation (Liu et al., 2022). A rapid initial response to stress involves a general suppression of protein synthesis, which leads to growth inhibition (Good and Zaplachinski, 1994; Dhindsa and Cleland, 1975). Concurrently, protein quality-control systems, particularly chaperone-mediated folding and endoplasmic reticulum–associated processing pathways, are transcriptionally induced to stabilize stress-sensitive nascent polypeptides and avert deleterious

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https://doi.org/10.1016/j.jplph.2026.154695
Received 22 August 2025; Received in revised form 2 December 2025; Accepted 9 January 2026
Available online 9 January 2026
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conformational deviations during co- and post-translational folding, thereby preserving proteostasis (Liu and Howell, 2010). As stress severity increases, energy metabolism becomes progressively impaired, affecting carbone allocation, lipid metabolism and photosynthetic efficiency. Consequently, the stress response involves a series of gradual and complex metabolic changes. The early stages of cold stress show a remarkable enrichment of cellular components, biological processes and molecular functions (Sinha et al., 2015; Jurkow et al., 2019). Transcriptomic analyses support these observations by identifying the upregulation of signalling pathways related to flavonoid biosynthesis, plant hormone and fructose metabolism at the onset of cold stress (Liu et al., 2024). In contrast, the signalling pathways related to amino sugar and nucleotide sugar metabolism, alanine and protein export as well as aspartate and glutamate metabolism increasingly change in the later stages (Hao et al., 2018). In addition to the changes in sugar metabolism, cold stress significantly disrupts chlorophyll biosynthesis, leading to an imbalance between photosystem I (PSI) and photosystem II (PSII). Research has shown that exposure to low temperatures downregulates key proteins involved in light-harvesting complexes, which in turn impairs the overall efficiency of photosynthesis (Adam and Murthy, 2014). PSII in particular is more severely damaged than PSI under cold stress. The reduction in chlorophyll content combined with the yellowing of plant tissue indicates that cold stress affect biosynthetic pathways and further impairs the plant's ability to effectively capture and utilise light (Chauhan et al., 2023).

While the genetic diversity of kale (*Brassica oleracea* L. var. *acephala*) has been extensively characterized using agro-morphological and SSR markers (Pipan et al., 2024; Malik et al., 2024; Maggioni et al., 2014), less is known about the biochemical changes it undergoes under abiotic stress conditions such as low temperatures. Previous research mainly focussed on the effects of various abiotic stress factors such as drought, and light (Ben Ammar et al., 2023; Arena et al., 2024) while the effects of cold temperatures on kale have been neglected. While pigments such as chlorophyll and carotenoids are known for their important role in plant health and consumer appeal, there is little data on how frost affects the content of pigments, glucosinolates and sugars in kale. Understanding the nutritional profiles of these kale varieties is critical to improving food security and promoting sustainable agricultural practises, particularly in light of evolving environmental challenges.

This study investigates the variations in the biochemical profiles of 26 kale accessions before and after short-term exposure to frost in open field, an aspect not previously reported in a combined analysis. The main objectives were to determine the influence of genetic diversity on the major biochemical compounds, sugars (sucrose, glucose, fructose, galactose, total sugars), glucosinolates (aliphatic, indole, aromatic, total glucosinolates) and pigments (chlorophyll *a*, chlorophyll *b*, total chlorophylls, total carotenoids) in technologically mature leaves (defined as the third to fifth fully expanded leaf below the apical meristem, corresponding to a metabolically stable phase). In addition, the effect of frost exposure on the modulation of these metabolites was evaluated for each accession. The results of this study contribute to efforts to improve the genetic resources of kale, with direct applications in breeding for improved frost tolerance, nutritional quality and food safety. In addition, the findings provide valuable insights into the conservation and sustainable utilisation of kale biodiversity, particularly in the south-eastern European region.

2. Materials and methods

2.1. Plant material and experimental design

Building on the comprehensive agro-morphological and molecular characterization of *Brassica oleracea* var. *acephala* accessions by Pipan et al. (2024), the present study advances this work through biochemical profiling conducted under open-field conditions with naturally occurring frost events. The experimental design was intentionally developed to overcome the constraints of controlled-environment studies, enabling the *in situ* investigation of cold-induced metabolic responses under agronomically relevant conditions. The plant material used in this study consisted of 26 accessions of *B. oleracea* var. *acephala.* Of these, 23 accessions were originated from two national plant genebanks (six from Croatia and 17 from Bosnia and Herzegovina). The other three were commercial varieties acquired from seed companies, i.e. Semenarna Ljubljana and Semina Royal Seeds from Slovenia, and Franchi Sementi from Italy. A summary of the available passport data for the analysed accessions can be found in Table S1. Plant propagation and field trials were conducted during the growing season from March to December 2019 in the experimental fields of the Agricultural Institute of Slovenia in Jablje (304 m a.s.l.; 46.151°N, 14.562°E). Sowing of kale seeds was performed in early March under greenhouse conditions, using 104-cell polystyrene trays filled with a commercial peat substrate (Potgrond P, Klasmann).At the time of transplanting in May, plants were at the young vegetative stage, characterized by the development of 6–8 true leaves, as defined in the Descriptors for *Brassica* and *Raphanus* (IBPGR, 1990). The transplanting was manually established on polyethylene-covered ridges equipped with a drip irrigation system in an open field. The experiment was established in double-row strips, with plants spaced 50 × 50 cm apart in four biological replicates (i.e. four plants per accession). Prior to ploughing in autumn, the soil was fertilised with 20 t/ha of cattle manure. During the growth period, only plant protection against diseases and pests was applied, no chemical pesticides or fungicides were applied during the experimental period. Manual weeding was conducted regularly, and pest management followed principles of integrated pest management (IPM) in alignment with organic production guidelines. The kale accessions were cultivated according to the established methodology, which included regular weeding and irrigation.

At the time of cold exposure, *Brassica oleracea* var. *acephala* plants were at the mature vegetative stage (8–10 true leaves), corresponding to technological maturity used for biomass and metabolomic assessments. Leaf samples were collected before (4th October) and after (11th December) natural frost exposure, with three undamaged leaves per plant pooled per biological replicate. During the 37-day period, plants experienced four natural frost events, with canopy-level minimum temperatures ranging from −1.1 °C to −2.3 °C, recorded by field sensors (Fig. S1). These events were defined based on agro-meteorological criteria (temperatures <0 °C), with biological significance increasing below −2 °C. This temperature range is consistent with published thresholds known to induce cold-responsive metabolic adjustments in *B. oleracea* without causing irreversible damage (Steindal et al., 2015). Following collection, samples were immediately frozen in liquid nitrogen, freeze-dried (Christ Gamma 1–20), and ground (Retsch MM400; 30 Hz, 2 min). This natural field-based approach improves ecological relevance and supports reproducibility in cold stress studies.

2.2. Extraction and determination of sugars

Sugar extraction and quantification were performed according to the method described by Simkova et al. (2023) with minor modifications. 0.05 g of homogenised freeze-dried leaf powder was extracted with 10 mL of double-distilled water. After a 30 min extraction at room temperature with frequent stirring, the extract was centrifuged for 10 min at 12,800×*g* (Eppendorf Centrifuge 5810 R, Hamburg, Germany). The supernatant was filtered through a 0.2 μm mixed cellulose ester filter (Macherey-Nagel; Düren, Germany) and poured into a vial before being analysed using a high-performance liquid chromatography system (HPLC; Thermo Scientific, Finnigan Spectra System, Waltham, MA, USA). A sample volume of 20 μL was used for the analysis that was performed using a Phenomenex Rezex RCM-Monosaccharide column with a flow rate of 0.6 mL/min at 65 °C. Double-distilled water was used as the mobile phase. A refractive index (RI) detector was used to identify sugars. The carbohydrates were identified and quantified by comparing the retention times and concentrations of the corresponding external

standards. The results are expressed in mg/g dry weight (DW) (Table S3).

2.3. Extraction, identification and quantification of glucosinolates

For the analysis of individual glucosinolates, 0.2 g of homogenised freeze-dried leaf powder was extracted with 2.5 mL of boiled 70 % methanol and placed in an oven at 75 °C for 10 min, according to the standardised method (ISO 9167, 2019) for desulfo-glucosinolates. The samples were centrifuged for 5 min at room temperature at 10,000 rpm (Eppendorf Centrifuge 5810 R). The supernatant was transferred to a second centrifuge and the samples were re-extracted with 2.5 mL of boiling 70 % methanol. This procedure was repeated. The supernatant from the second repetition was combined with the first supernatant, resulting in a total of 5 mL of supernatant. Samples were then transferred to previously prepared DEAE Sephadex A-25 columns (500 μL). The columns were then washed twice with 1 mL sodium acetate buffer solution (0.02 M at pH 4.0). Desulfation was achieved by applying 75 μL of the sulfatase enzyme solution to the columns, which were then left overnight at room temperature. The next day, the columns were washed twice with 750 μL of double-distilled water and the liquid that flowed through the column was collected in coils. The desulfo-glucosinolates were analysed using an HPLC system (HPLC; Thermo Scientific, Finnigan Spectra System, Waltham, MA, USA) with a PDA detector (photodiode array) at a wavelength of 229 nm. A Gemini C18 column (150 × 4.6 mm, 3 μm) was kept at 30 °C. The mobile phases used were A (100 % H_2O) and B (20 % acetonitrile in H_2O), according to the gradient described by Jakopič et al. (2016). Desulfo-glucosinolates were identified by comparison with the retention times of the standards using the LTQ XL mass spectrometer (Thermo Scientific, Finnigan Spectra System, Waltham, MA, USA) based on their fragmentation pattern. The sample injection was 10 μL and the other chromatographic conditions were the same as described for HPLC analyses. The mass spectrometer operated in positive ion mode with electrospray ionisation (ESI). The capillary temperature was 250 °C, the sheath gas 20 units and the auxiliary gas 8 units. The source voltage used was 4 kV, with an *m/z* scan of 115–1600. The content was calculated using the calibration curves of the corresponding standards. The results were expressed in mg/g DW (Table S4). For glucosinolates without available standards, the results were expressed in mg equivalents of sinigrin, glucobrassicanapin or glucobrassicin per g DW as commonly practiced in glucosinolate analysis (Aires et al., 2013).

2.4. Determination of chlorophylls and carotenoids content

The extraction of photosynthetic pigments was performed according to the protocol described by Monni et al. (2001). 30 mg of homogenised freeze-dried leaf powder was extracted with 5 mL of an 80 % acetone solution. The centrifuges were covered with rubber caps, the level of acetone was marked, covered with aluminium foil and incubated in a refrigerator at 4 °C overnight. The next day, acetone was added up to the mark. The samples were first vortexed and then centrifuged for 2 min at 2500 rpm. The absorbance was measured with a spectrophotometer (Shimadzu, UV-1800) at wavelengths of 470, 647 and 664 nm, using an 80 % acetone solution as a control. From the measured absorbances at different wavelengths, the concentration of chlorophyll *a* ($C_a = 11.24 \times A_{664} – 2.79 \times A_{647}$), chlorophyll *b* ($C_b = 21.50 \times A_{647} – 5.10 \times A_{664}$), total chlorophyll ($C_{a+b} = 7.15 \times A_{664} + 18.71 \times A_{647}$) and total carotenoids ($C_{x+b} = ((1000 \times A_{470} – 1.82 \times C_a – 95.15 \times C_b)/225)$) were calculated as described by Lichtenthaler and Buschmann (2001) and Lichtenthaler (1987) and the results were expressed in mg/g DW (Table S5).

2.5. Data analysis

All statistical analyses and visualisations were carried out using the R programming language (version 4.4.1, R Core Team, 2020). First, the *pastecs* package (Grosjean et al., 2018) was used to calculate descriptive statistics, including the mean, standard error (SE), maximum (Max) and minimum (Min) and coefficient of variation (CV) for each biochemical trait to summarise the variability between accessions before and after frost. One-way and two-way analyses of variance (ANOVA) were then performed using the *stats* package to assess the main effects of frost treatment, kale accessions and their interaction on the biochemical traits. Pairwise post-hoc comparisons were performed using Tukey's honest significant difference (HSD) test in the *stats* package (R Core Team, 2020), with the significance threshold set at $p < 0.05$. To investigate the overall effect of frost on the biochemical composition of kale and accession-specific variation, boxplots were generated using the *ggplot2* package (Wickham, 2016) to visualise the variation in total sugars, glucosinolates, total chlorophyll and total carotenoids across frost treatments and accessions. Pearson's correlation coefficients were calculated using the *cor* function from the *stats* package to analyse the relationships among sugars, glucosinolates and pigments before and after frost. These relationships were visualised using heatmaps created with the *corrplot* package (Wei and Simko, 2017). Principal component analysis (PCA) was then performed using the *FactoMineR* package (Lê et al., 2008), with visualisation facilitated by the *fctoextra* package (Kassambara and Mundt, 2017). PCA was used to reduce the dimensionality of the dataset and identify the key variables contributing to the biochemical variation between accessions. The first two principal components, which captured most of the variance, were used to group accessions based on their metabolic profiles. Biplots were created to illustrate the separation between the before and after frost groups and to highlight accession-specific metabolic responses.

3. Results

3.1. Quantitative analysis

Before frost, sucrose content varied between accessions, ranging from 34.66 mg/g DW in Accession_19 to 102.29 mg/g DW in Accession_26, with a mean of 64.29 mg/g DW and a coefficient of variation (CV) of 35.15 % (Table 1). This moderate variability indicates a consistent distribution of sucrose content among the kale accessions. In contrast, sucrose accumulation increased significantly after frost, ranging from 130.55 mg/g DW in Accession_24 to 264.76 mg/g DW in Accession_4, with a mean of 202.53 mg/g DW and a reduced CV of 24.26 % (Table 1). Accession_4 emerged as the genotype showing the greatest metabolic adjustment, exhibiting a markedly higher sucrose accumulation after frost. In contrast to sucrose, glucose levels decreased significantly after frost. Contents before frost ranged from 56.19 mg/g DW in Accession_6 to 115.46 mg/g DW in Accession_21, with a mean of 79.73 mg/g DW and a CV of 22.92 %, indicating moderate variability in glucose levels between accessions. After frost, glucose content decreased significantly and ranged from 20.37 mg/g DW in Accession_8 to 59.58 mg/g DW in Accession_3, with a reduced mean of 41.08 mg/g DW and an increased CV of 30.55 %. The higher CV after frost indicates greater variability in the response of glucose metabolism between accessions to cold stress. The galactose content was relatively low under both conditions. Before frost, the galactose contents ranged from 4.68 mg/g DW in Accession_2 to 9.30 mg/g DW in Accession_8, with a mean of 6.34 mg/g DW. After frost, the galactose content increased, ranging from 5.35 mg/g DW in Accession_27 to 14.00 mg/g DW in Accession_10, with a higher mean of 9.50 mg/g DW. Before frost, fructose varied from 61.97 mg/g DW in Accession_10 to 133.54 mg/g DW in Accession_2, with a mean of 95.56 mg/g DW and a CV of 20.21 %, indicating relatively consistent fructose levels between accessions. After frost, fructose decreased and ranged from 40.9 mg/g DW in Accession_1 to 99.07 mg/g DW in Accession_9, with a lower mean of 69.56 mg/g DW and an increased CV of 27.79 %. The total sugar content showed a remarkable contrast before and after frost conditions, as shown in Fig. 1A. Before frost, total sugar content ranged from 201.17 mg/g DW in Accession_19 to 338.10 mg/g

Table 1
Descriptive statistics of the analysed biochemical compounds under cold stress conditions.

Biochemical compounds (mg/g dry weight)	Before frost				After frost			
	Min	Max	Mean ± SE	CV (%)	Min	Max	Mean ± SE	CV (%)
Sucrose	34.66	102.29	64.29 ± 16.71	35.15	130.55	264.76	202.53 ± 36.85	24.26
Glucose	56.19	115.46	79.73 ± 14.50	22.92	20.37	59.58	41.08 ± 9.07	30.55
Galactose	4.68	9.30	6.34 ± 0.96	23.17	5.35	14.00	9.50 ± 2.38	34.98
Fructose	61.97	133.54	95.56 ± 15.06	20.21	40.90	99.07	69.56 ± 14.53	27.79
Total sugars	201.17	338.10	245.92 ± 42.05	17.10	254.20	388.28	322.68 ± 54.92	17.02
Glucoiberin	0.00	0.17	0.039 ± 0.036	92.74	0.00	1.21	0.31 ± 0.27	105.60
Sinigrin	0.03	6.13	0.76 ± 1.17	157.60	0.00	7.10	2.57 ± 1.46	68.81
Unknown 1	0.03	0.11	0.07 ± 0.03	49.26	0.00	0.06	0.03 ± 0.01	53.76
Unknown 2	0.00	0.22	0.10 ± 0.05	54.65	0.07	0.22	0.14 ± 0.04	40.72
4-hydroxyglucobrassicin	0.00	0.26	0.11 ± 0.06	59.19	0.08	0.24	0.16 ± 0.05	37.52
Gluconapin	0.02	0.18	0.05 ± 0.04	100.17	0.03	0.51	0.20 ± 0.16	108.60
Glucobrassicanapin	0.00	8.72	1.02 ± 1.90	197.74	0.00	2.03	0.42 ± 0.46	118.83
Unknown 3	0.00	0.17	0.07 ± 0.05	83.38	0.00	0.13	0.04 ± 0.03	92.21
Glucobrassicin	0.65	13.93	5.99 ± 3.86	69.77	1.55	7.43	4.39 ± 1.50	44.89
4-methoxyglucobrassicin	0.00	1.16	0.08 ± 0.24	299.97	0.00	0.14	0.02 ± 0.03	207.21
Gluconasturtiin	0.21	3.24	0.97 ± 0.68	82.24	0.19	1.72	0.79 ± 0.35	62.86
Neoglucobrassicin	0.00	0.535	0.018 ± 0.087	492.99	0.00	0.11	0.002 ± 0.013	655.89
Total glucosinolates	3.07	18.49	9.27 ± 4.65	54.78	5.16	14.26	9.10 ± 2.17	31.85
Aliphatic glucosinolates	4.73	68.72	20.70 ± 17.64	89.07	13.27	74.30	38.56 ± 14.60	44.08
Indolic glucosinolates	15.93	85.55	67.38 ± 17.61	28.25	15.11	82.83	51.95 ± 13.79	31.25
Aromatic glucosinolates	3.79	28.04	11.92 ± 5.80	59.63	3.74	18.49	9.49 ± 4.31	64.64
Chlorophyll *a*	1.52	4.04	2.81 ± 0.65	28.23	0.88	3.30	1.90 ± 0.52	33.65
Chlorophyll *b*	0.59	1.83	1.19 ± 0.33	34.22	0.28	1.78	0.84 ± 0.30	43.63
Total chlorophylls	2.11	5.87	4.01 ± 0.98	29.86	1.16	5.08	2.73 ± 0.82	36.51
Total carotenoids	0.33	0.79	0.53 ± 0.11	24.79	0.15	0.63	0.37 ± 0.09	31.65

SE, standard error of the mean; CV, coefficient of variation.

DW in Accession_21, with a mean of 245.92 mg/g DW and a CV of 17.10 % (Table 1). This high variability indicates considerable differences in sugar accumulation between the leaf samples. After frost, the total sugar content varied between 254.20 mg/g DW in Accession_24 and 388.28 mg/g DW in Accession_1, with a mean of 322.68 mg/g DW and a similar CV of 17.02 %.

Analysis of glucosinolate profiles before and after frost conditions revealed significant variability in individual and total glucosinolate contents (Table 1). Glucoiberin showed a significant increase from 0.039 mg/g DW (CV = 92.74 %) to 0.31 mg/g DW (CV = 105.60 %) before and after frost, respectively. Similarly, sinigrin showed high variability with a mean of 0.76 mg/g DW (CV = 157.60 %) and 2.57 mg/g DW (CV = 68.81 %) before and after frost, respectively. Indolic glucosinolates, especially glucobrassicin, dominated the profile with a mean of 5.99 mg/g DW (CV = 69.77 %) before frost, which decreased to 4.39 mg/g DW (CV = 44.89 %) after frost stress. Interestingly, the proportion of aliphatic glucosinolates increased significantly after frost, with a mean of 38.6 %, compared to 20.7 % before frost. The total glucosinolate content remained relatively comparable before and after frost conditions, but notable differences were observed between accessions, as shown in Fig. 1B. Before frost, the total glucosinolate content ranged from 3.07 mg/g DW in Accession_3 to 18.49 mg/g DW in Accession_25. However, after frost, the highest glucosinolate content was observed in Accession_3, which showed a significant increase to 14.62 mg/g DW, while the lowest content was observed in Accession_27 (5.16 mg/g DW).

The chlorophyll and carotenoid content were also quantitatively assessed as part of the investigation. Before frost, chlorophyll *a* varied between accessions, ranging from 1.52 mg/g DW in Accession_1 to 4.04 mg/g DW in Accession_25, with a mean of 2.81 mg/g DW and a CV of 28.23 %. However, after frost, the chlorophyll *a* content decreased significantly, with a mean of 1.90 mg/g DW. The highest chlorophyll *a* content after cold stress was found in Accession_2 (3.03 mg/g DW). Chlorophyll *b* content varied before frost, ranging from 0.59 mg/g DW in Accession_1 to 1.83 mg/g DW in Accession_25, with a mean of 1.19 mg/g DW and a CV of 34.22 %. These variabilities reflect the differences in chlorophyll *a* and chlorophyll *b* content between accessions under optimal growth conditions. After frost, chlorophyll *b* decreased, with values ranging from 0.28 mg/g DW in Accession_8 to 1.78 mg/g DW in Accession_2. The mean content after frost was 0.84 mg/g DW, accompanied by a higher CV of 43.63 %. As shown in Fig. 2A, total chlorophylls decreased after frost. Accession_8 had the lowest total chlorophylls under both conditions, with 2.11 mg/g DW before frost and 1.16 mg/g DW after frost. In contrast, the highest total chlorophylls were observed in Accession_25 before frost (5.87 mg/g DW) and in Accession_2 after frost (5.08 mg/g DW). Total carotenoids showed a slight decrease after cold stress with a mean of 0.37 mg/g DW and a CV of 31.65 %, compared to 0.53 mg/g DW and a CV of 24.79 % before frost. As seen in Fig. 2B, the highest carotenoids were observed in Accession_2 under both conditions.

3.2. Insights from correlation analysis

The Pearson correlation matrix showed significant shifts in the relationships between the biochemical profiles of sugars, glucosinolates and pigments before and after frost, providing valuable insights into the metabolic adaptations induced by frost stress (Fig. 4). Before frost, sucrose had a negative correlation with total sugars (r = −0.40), which turned into a positive correlation (r = 0.66) after frost. This indicates a possible redistribution of carbohydrate resources in response to abiotic stress. A similar trend was observed between sucrose and aliphatic glucosinolates, glucoiberin, with a negative correlation before frost (r = −0.40) that turned positive after frost (r = 0.12), indicating a shift in the interplay between sugar and glucosinolate metabolism during cold stress. Before frost, sinigrin showed a negative correlation with total sugars (r = −0.54), which transitioned to a positive but weaker correlation after frost (r = −0.23). Before frost, 4-methoxyglucobrassicin and its precursor, 4-hydroxyglucobrassicin, showed a negative correlation (r = −0.45), suggesting a regulatory mechanism whereby the synthesis of one glucosinolate can inhibit the other. This negative correlation is disturbed after frost, indicating a breakdown in normal metabolic regulation. The aromatic glucosinolate, gluconasturtiin showed a positive correlation with fructose before frost (r = 0.41). However, this correlation was reversed under cold stress (r = −0.41), indicating a

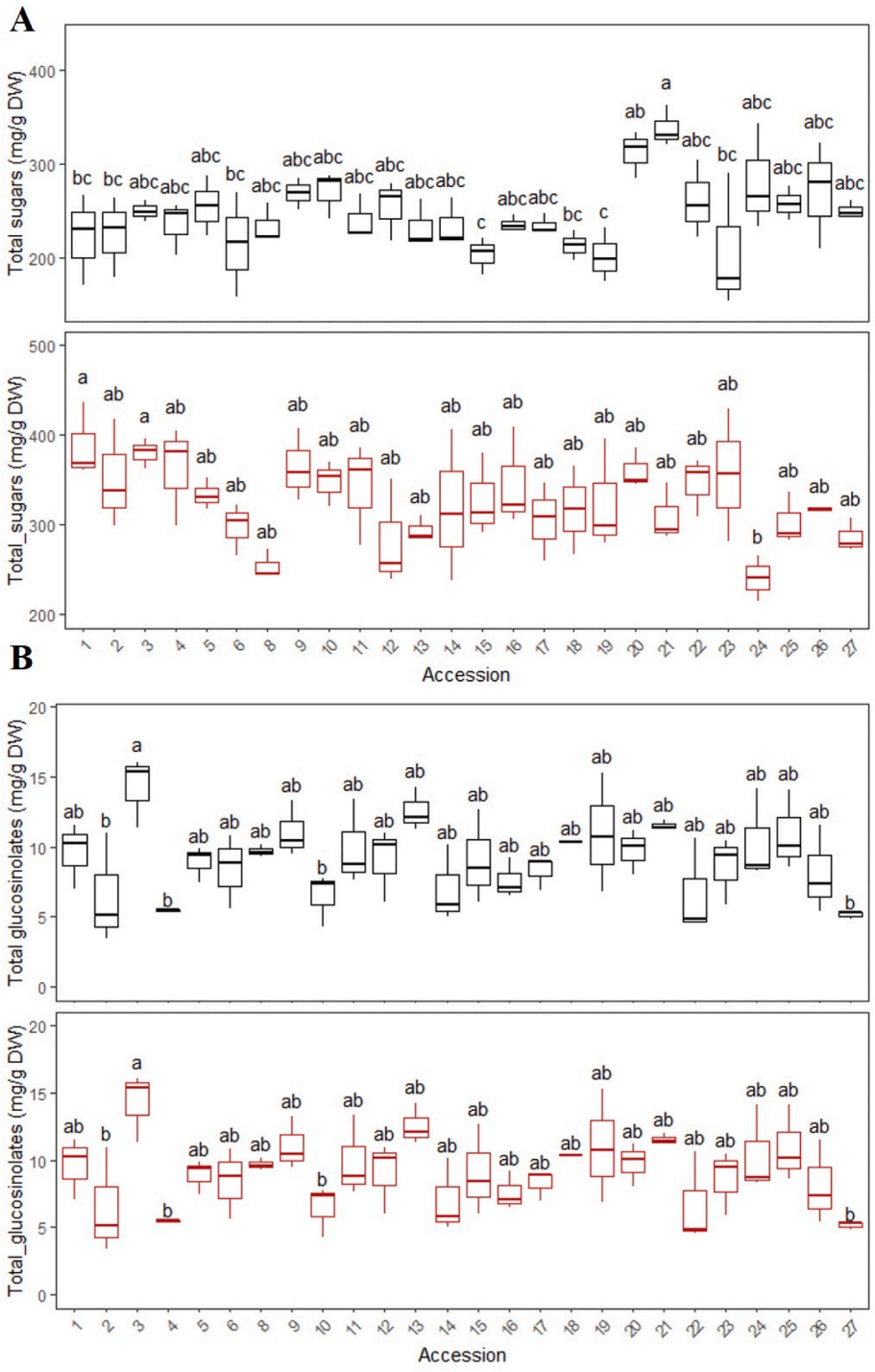

Fig. 1. Total sugar content (A) and total glucosinolates (B) in 26 kale accessions. The boxplot compares before frost (black) and after frost conditions (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

significant change in the metabolic interaction between aromatic glucosinolates and sugars after frost. Regarding glucose and photosynthetic pigments, a positive correlation between glucose and chlorophyll *a*, chlorophyll *b* and total carotenoids was observed before frost. These correlations disappeared under cold stress. Interestingly, the relationships between the photosynthetic pigments themselves remained positive under both conditions, while the correlations between glucose and pigments were somewhat stronger after frost.

3.3. *Effect of the experimental conditions in the biochemical traits*

The results showed that the factors frost, accession and their interaction contribute differently to the explained variance of the analysed biochemical parameters (Table 2, Fig. 3). For sugars, frost contributed significantly to the variance, especially for sucrose and glucose, which accounted for about 60 % of the total variance, while fructose and total sugar content were dominated by accessions, indicating a significant genetic influence on these traits. The interaction effect (frost × accession) was moderate for individual sugars, while for the total sugar

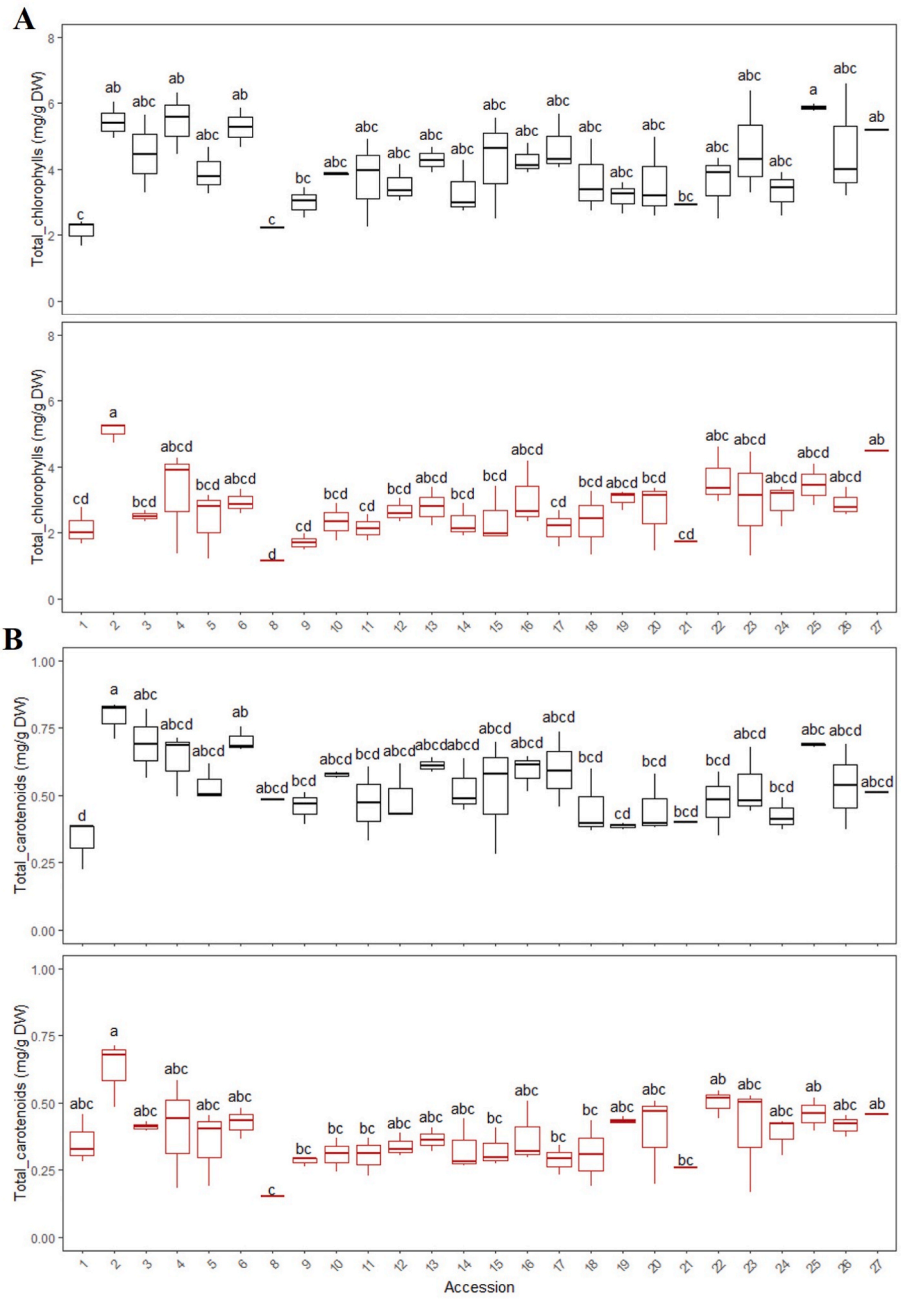

Fig. 2. Total chlorophyll content (A) and total carotenoids (B) in 26 kale accessions. The boxplot compares before frost (black) and after frost conditions (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

content had no significant effect, suggesting a combined influence of genetic and environmental factors on sugar metabolism. The variance in glucobrassicin, 4-methoxyglucobrassicin and total glucosinolates was primarily attributed to accession, emphasising genetic control over indolic glucosinolates. Frost had a lesser effect, but remained notable for certain glucosinolates such as aliphatic glucosinolate sinigrin (Fig. 3). The interaction effect was particularly pronounced for certain glucosinolates (i.e. neoglucobrassicin, glucobrassicanapin), reflecting the complexity of genotype-environment interactions. Chlorophyll *a* and total carotenoids showed a balanced contribution of the factors frost and accession, each explaining about 30 % of the total variance (Fig. 3). In contrast, chlorophyll *b* and chlorophyll-to-carotenoid ratio showed a higher residual variance, indicating a limited influence of the assessed factors. However, the interaction of the factors frost and accession is not significant for chlorophyll *a*, chlorophyll *b* and total chlorophyll (Table 2).

3.4. Multivariate insights from PCA

The PCA biplot offers an integrated view of the metabolic adjustments of kale accessions before and after frost exposure (Fig. 5). The first two principal components, Dim1 (31.3 %) and Dim2 (16.7 %), together explain 48.0 % of the total variance. A metabolic differentiation between non-stressed and frost-exposed samples is mainly captured along Dim1, where frost-treated accessions (red circles) consistently display more positive score values, although a degree of overlap remains. This axis reflects an inverse metabolic trend between aliphatic glucosinolates and carbohydrate-related metabolites (sucrose, galactose, total sugars) on one side and photosynthetic pigments together with aromatic and indolic glucosinolates on the opposite side. The proximity of non-

Table 2
Analysis of variance showing the effects of frost, accessions and their interactions.

Parameter	Frost		Accession		Frost*Accession	
	F-value	Sig.	F-value	Sig.	F-value	Sig.
Sucrose	780.37	***	2.47	***	2.34	**
Glucose	316.35	***	2.49	***	2.05	**
Galactose	40.16	***	0.95	ns	1.72	*
Fructose	105.29	***	3.08	***	2.01	**
Total sugars	153.77	***	1.95	*	1.50	ns
Glucoiberin	144.30	***	4.74	***	3.81	***
Sinigrin	221.99	***	9.75	***	2.26	**
Unknown 1	106.38	***	4.20	***	1.93	*
Unknown 2	32.14	***	5.70	***	2.08	**
4-hydroxyglucobrassicin	48.15	***	5.63	***	2.44	***
Gluconapin	54.06	***	3.01	***	1.92	*
Glucobrassicanapin	26.43	***	12.79	***	15.86	***
Unknown 3	28.41	***	10.06	***	1.58	ns
Glucobrassicin	5.54	*	7.17	***	3.45	***
4-methoxyglucobrassicin	32.23	***	21.88	***	20.37	***
Gluconasturtiin	3.85	ns	4.46	***	2.88	***
Neoglucobrassicin	67.88	***	84.87	***	86.69	***
Total glucosinolates	2.65	ns	6.39	***	4.56	***
Aliphatic glucosinolates	188.53	***	10.78	***	4.49	***
Indolic glucosinolates	57.92	***	13.53	***	2.73	***
Aromatic glucosinolates	8.75	**	3.26	***	2.02	**
Chlorophyll *a*	117.67	***	6.13	***	1.14	ns
Chlorophyll *b*	63.01	***	5.88	***	1.09	ns
Total chlorophylls	96.56	***	5.96	***	1.09	ns
Total carotenoids	108.24	***	4.38	***	1.73	*
Chlorophyll *a*/*b* ratio	0.42	ns	3.28	***	1.82	*
Chlorophylls/carotenoids ratio	1.41	ns	6.34	***	3.14	***

ns, not significant; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$, respectively.

stressed accessions to aliphatic glucosinolates and sugars suggests that these compounds support metabolic homeostasis under favourable conditions. In line with this, the uncharacterised glucosinolate Unknown 2 aligns with these metabolites, which may indicate a potential role in metabolic stability during optimal growth. Conversely, frost-exposed accessions are positioned closer to photosynthetic parameters and aromatic/indolic glucosinolates, supporting a Dim1-dependent metabolic reconfiguration associated with cold stress. Unidentified glucosinolates (Unknown 1 and Unknown 3) are also located near aromatic and indolic glucosinolates, suggesting they may contribute to stress-related metabolic pathways, although their precise functions warrant further investigation. Accession_27 occupies an intermediate position between the two treatment groups, indicating a mixed metabolic signature that might reflect an intermediate or accession-specific response rather than a typical reaction to frost. In contrast, Accession_8 is located at an extreme negative value of Dim2, while its Dim1 score remains close to zero. This position does not align with the frost-associated variation captured by Dim1. Instead, it likely reflects an accession-specific metabolic configuration, consistent with Dim2 representing intrinsic variability rather than frost-driven metabolic responses.

4. Discussion

The results presented show significant changes in secondary metabolites in response to frost stress, highlighting both the adaptive mechanisms that plants use under cold conditions and the variability of responses between different leafy kale accessions. Sugar metabolism plays a crucial role in the response of plants to various abiotic stress factors, especially in improving tolerance to osmotic challenges such as drought, salinity and frost (Zuther et al., 2024). The variation in sugar content before and after frost indicates that cold stress leads to shifts in carbohydrate metabolism, with distinct accumulation patterns observed for different metabolites. Its accumulation probably helps to stabilize cell structures, maintain osmotic balance and mitigate the harmful effects of frost (Orzechowski et al., 2021). The lower variability before frost suggests a more uniform response of the different accessions to cold stress, reinforcing the idea that sucrose may act as a stress-responsive key metabolite to improve cold tolerance (Jeandet et al., 2022). In this context, kale Accession_4 with the highest sucrose content, can be considered one of the most frost-tolerant accessions in this study. The significant decrease in glucose content after frost may reflect a strategy of conserving glucose for critical metabolic functions and possibly reallocating resources for protective measures such as sucrose accumulation (Sami et al., 2016). Both galactose and fructose showed relatively low levels before frost, but they responded differently to cold stress. Galactose levels increased after frost, indicating a shift in carbohydrate metabolism possibly related to the synthesis of protective compounds. However, the increased variability in galactose content between accessions after frost suggests that different accessions may use galactose differently in response to stress. In contrast, fructose content decreased after frost. This decrease suggests that cold stress may affect the biosynthesis or accumulation of fructose, possibly due to changes in metabolic pathways (Bogdanović et al., 2008). The higher variability in fructose levels after frost reflects the diverse responses among accessions to cold stress, with some accessions showing higher decrease in fructose than others (Tarkowski and Van den Ende, 2015). The differences in sugar metabolism between accessions under cold stress emphasise the importance of understanding how different accessions respond to environmental stressors such as frost (Gupta and Kaur, 2005). Kale Accession_4 and Accession_1 were found to be the most efficient in terms of sucrose and total sugar accumulation, making them potential candidates for breeding programmes to improve frost tolerance. These accessions show the ability to consistently accumulate sugars under cold stress, suggesting that they have inherent mechanisms to mitigate frost-induced damage. On the other hand, kale Accession_8, which had lower glucose content after frost, may have a different stress response strategy, possibly by reallocating resources to other metabolic pathways. The lower variability and higher mean sugar content after frost suggest that cold stress leads to more uniform sugar accumulation, possibly representing an adaptive response to minimise the risk of frost damage.

The variability in glucosinolate content observed before and after frost illustrates the complex biochemical responses of plants to environmental stress. Frost exposure led to a significant increase in aliphatic glucosinolates such as glucoiberin and gluconapin, suggesting that they enhance the plant's defence mechanisms against cold stress. For example, the mean of gluconapin content increased from 0.05 ± 0.04 mg/g DW before frost to 0.20 ± 0.16 mg/g DW after frost, supporting the idea that aliphatic glucosinolates are particularly sensitive to frost and may play a protective role in cell function or stress signalling. These results are consistent with previous studies of Hahn et al. (2023) who found that cold acclimatisation caused a substantial increase in aliphatic glucosinolates in curly and lacinato kale, with levels of glucoraphanin, gluconapin, progoitrin and sinigrin increasing by more than 200 % compared to those observed under warm conditions. This pronounced accumulation supports the hypothesis that aliphatic glucosinolates play a crucial role in adaptation to temperature change (Rao et al., 2021; Justen and Fritz, 2013). Conversely, indolic glucosinolates, such as glucobrassicin, decreased after frost, with levels dropping from 5.99 ± 3.86 mg/g DW to 4.39 ± 1.50 mg/g DW. This decrease indicates a possible trade-off in metabolic allocation, where the plant prioritizes aliphatic glucosinolate biosynthesis over indolic pathways under cold conditions as reported by Steindal et al. (2015). Despite the reduction, indolic glucosinolates remained the predominant group in the glucosinolate profile, which is consistent with results in kale, where indolic glucosinolates are known to dominate, as reported in the literature (Ferioli et al., 2013; Velasco et al., 2007; Korus et al., 2014; Ljubej et al., 2021). This suggests that although aliphatic glucosinolates respond dynamically to frost, the plant retains a baseline level of indolic

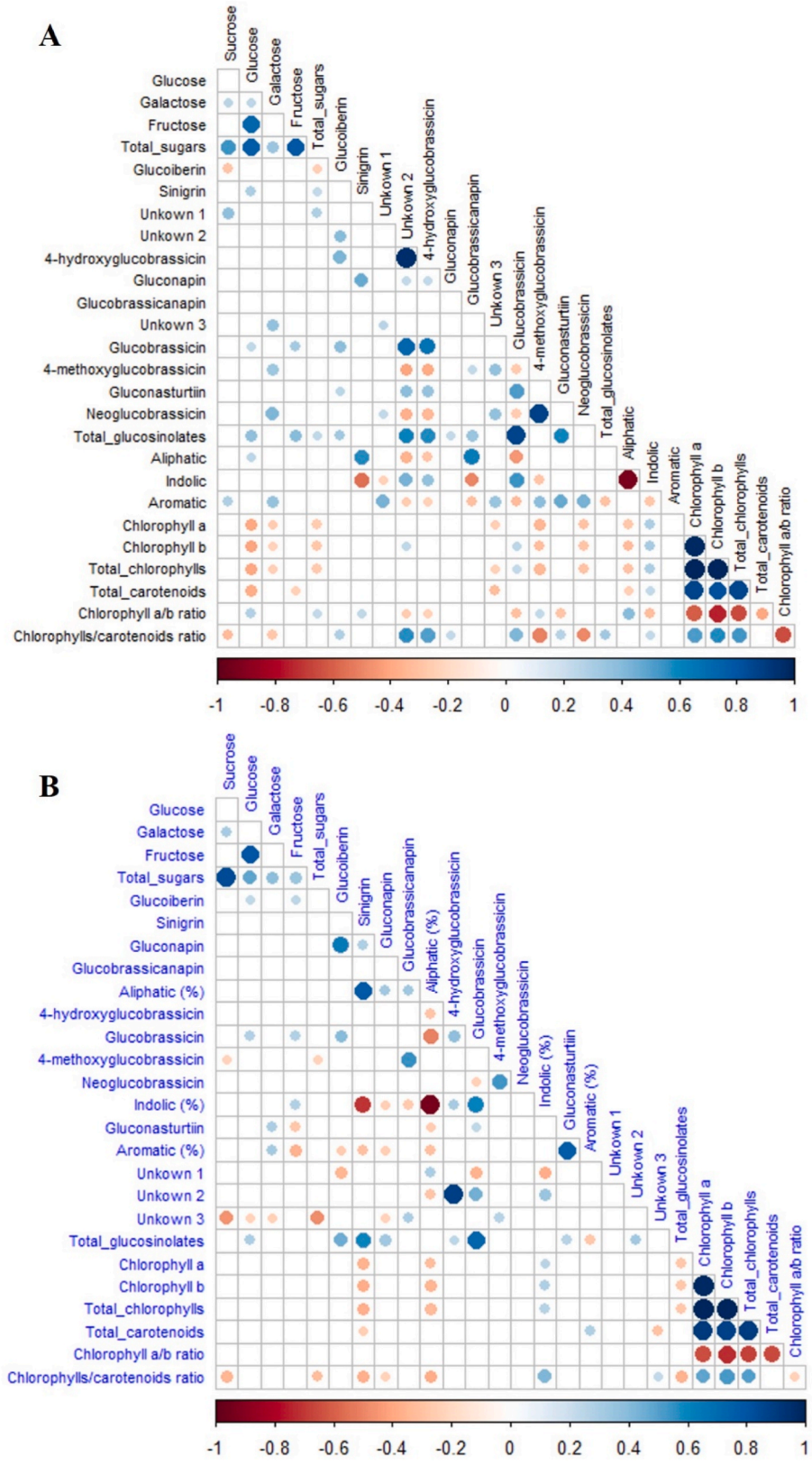

Fig. 3. Pearson's correlation matrices for the conditions before frost (A) and after frost (B).

glucosinolates, possibly for other physiological or ecological functions. Total glucosinolate content remained relatively stable before and after frost, suggesting that plant is able to maintain its overall defence potential by reallocating resources between glucosinolate groups. However, the significant variability observed, which is reflected in the high coefficients of variation (e.g. sinigrin, CV = 157.60 % and 68.81 % before and after frost, respectively), indicates genetic and environmental factors influencing the biosynthesis of glucosinolates. Thus, the

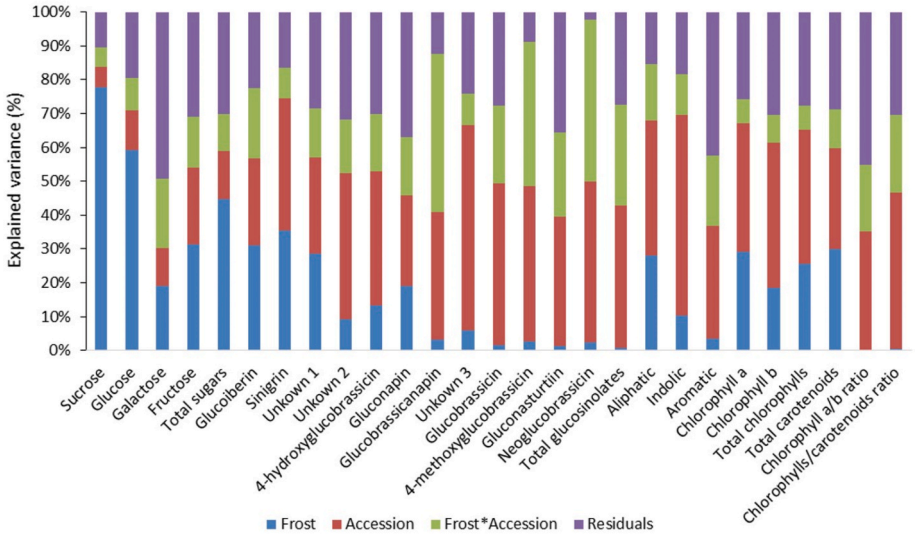

Fig. 4. Contribution of frost, accessions and their interactions to the total explained variance for the analysed biochemical parameters.

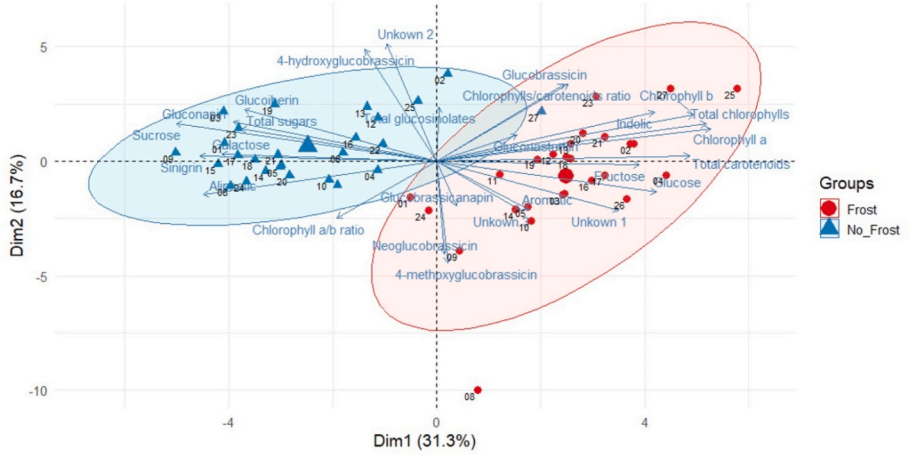

Fig. 5. PCA biplot of the variables for the conditions before frost (No_Frost) and after frost.

accumulation of specific glucosinolates is strongly influenced by the severity and duration of the abiotic stress as well as the developmental stage of the plant at the time of stress exposure (Martínez-Ballesta et al., 2013). Abiotic stress factors such as water deficit often lead to an increase in glucosinolate content due to their function as osmolytes in maintaining osmotic balance (Ben Ammar et al., 2023). As well, after frost, glucosinolate levels were generally higher than before frost, suggesting that frost exposure may differentially regulate their biosynthesis, degradation or accumulation as found in the study of Tong et al. (2021). This discrepancy emphasises the nuanced impact of low temperatures on glucosinolate metabolism, which appears to vary between kale varieties, highlighting the interplay of genetic predisposition (Ben Ammar et al., 2022) and environmental factors (Bohinc and Trdan, 2012). The observed variation in glucosinolate profiles, after frost exposure, underscore the metabolic plasticity of plants in adapting to extreme conditions. These changes reflect a complex trade-off between resource allocation, defence mechanisms and stress tolerance, in which metabolic adaptations may optimise survival and not only enhance defence. These findings provide a deeper insight into how plant secondary metabolism responds dynamically to environmental stressors and shapes both resilience and functional diversity across genotypes (Jakovljević et al., 2013).

With respect to the unknown glucosinolates, their probable structures were proposed based on the mass spectrometry data (Table S2). Unknown 1 (*m/z* 269, Rt = 0.64 min), which has a conspicuous fragmentation ion at *m/z* 137, indicates the loss of a glucose unit or a sulfonate group, suggesting that it may be glucoraphanin, an aliphatic glucosinolate commonly found in *Brassica* species. Unknown 2 (*m/z* 385, Rt = 11.65 min), with its major fragment at *m/z* 223 and minor fragments at *m/z* 205, 177 and 367, is not consistent with sinigrin or glucoiberin, as confirmed by comparison with standards. Instead, it is more likely to be glucoraphenin, a glucosinolate that also has a fragmentation pattern involving the loss of *m/z* 223 and other related fragments. Finally, Unknown 3 (*m/z* 421, Rt = 22.90 min), which has dominant fragments at *m/z* 259 and 393, is likely progoitrin or glucotropaeolin, which are known to produce similar fragmentation patterns. While these proposed identities agree well with the observed mass spectrometric data, further analysis, including comparison with additional known standards and advanced spectrometric techniques, is required to definitively confirm the exact structures of these unknown glucosinolates.

The overall reduction in chlorophyll content after frost indicates that the cold stress affects the photosynthetic machinery, possibly limiting the plant's ability to efficiently capture light for energy production (Orzechowski et al., 2021). This decrease in chlorophyll content is indicative of frost-induced stress, which can affect overall plant health

and productivity. The observed reduction in carotenoid content under frost conditions indicates a possible disruption of the plant's light-collecting efficiency, which may affect photosynthetic performance. As Šamec et al. (2022) found, exposure to low temperatures resulted in a significant increase in proline accumulation, a key marker of stress response, while simultaneously decreasing yield and carotenoid and flavonoid content of flat leaf kale sprouts. The reduction in carotenoid levels may reflect their active involvement in the oxidative defence or a metabolic reallocation toward alternative stress-response pathways, indicating a functional role within broader adaptive mechanisms against cold stress. The decrease in carotenoid content may reflect a trade-off in energy allocation, where the plant prioritizes protective responses such as proline accumulation to cope with the immediate stress, possibly at the expense of its photosynthetic apparatus and cellular integrity. While this adaptation is protective in the short term, it can compromise productivity in the long term by reducing the plant's ability to efficiently absorb light and photosynthesise. The decline in carotenoids after frost may therefore represent not only a direct response to oxidative stress, but also a broader strategy to maintain cellular homeostasis under challenging environmental conditions.

The observed shifts in the correlation patterns between different metabolites before and after frost reflect the metabolic adaptations that plants make in response to stress. The change from a negative to a positive correlation between sucrose and total sugars suggests that frost stress may trigger a redistribution of carbohydrates, possibly favouring energy sources for stress adaptation over normal growth (Seydel et al., 2022). Similarly, the reversal of the correlation between sucrose and glucoiberin indicates a frost-induced divergence in the metabolic pathways of sugars and glucosinolates, which could indicate a shift in resource allocation between primary and secondary metabolism under cold stress. The positive correlation between singrin and total sugars after frost points to a possible stress-induced diversion of carbon resources towards glucosinolate synthesis, suggesting an adaptive metabolic change (Vosnjak et al., 2021). The disappearance of the negative correlation between 4-methoxyglucobrassicin and 4-hydroxyglucobrassicin following frost exposure implies a disruption of the canonical regulatory circuitry controlling indole glucosinolate biosynthesis. This variation likely reflects a frost-induced metabolic reprogramming, wherein cellular resources are redistributed toward alternative stress-responsive pathways. In parallel, the altered correlation between gluconasturtiin and fructose further supports the hypothesis of a systemic reorganization of primary–secondary metabolic connectivity under frost conditions, consistent with broader metabolic network plasticity reported in abiotic stress contexts (Steindal et al., 2015). The emergence of a positive correlation between sinigrin and total sugars post-frost suggests an adaptive rerouting of carbon skeletons toward glucosinolate biosynthesis, potentially reflecting a shift in carbon allocation from growth-related processes to chemical defence, a mechanism previously associated with stress-induced metabolic prioritization (Martínez-Ballesta et al., 2013). Although the correlation between glucose and photosynthetic pigments was attenuated, a residual association persisted, indicating that photosynthetic metabolism remains partially coupled to carbohydrate dynamics. Moreover, the strengthened correlations among photosynthetic pigments themselves under frost stress may represent a compensatory adjustment aimed at preserving core photosynthetic capacity despite upstream metabolic perturbations. This adaptation highlights the plant's ability to reprogram its metabolism by prioritising primary metabolic processes while balancing the demands of secondary metabolism in response to frost stress (Koch, 1996). Environmental stresses, such as frost, pose major challenges to plant growth and metabolism. Understanding the relative contributions of environmental factors such as frost, genetic variation among accessions and their interactions is critical for the development of stress-resistant crops (Krasensky and Jonak, 2012).

The analysis emphasises the different effects of frost and genetic variation on the biochemical characteristics of plants. Frost significantly influences primary metabolites such as sugars, which is consistent with its role in stress-induced energy requirements and osmotic adjustment (Salam et al., 2023). Genetic variation represented by accessions plays a dominant role in secondary metabolites such as glucosinolates, which are essential for plant defence mechanisms (Ben Ammar et al., 2022). Interestingly, the minimal effect of frost on chlorophyll *b* and the chlorophyll-carotenoid ratio suggests that traits are stable under cold stress, possibly related to their essential role in photosynthetic resilience. The striking residual variance requires for further investigation of additional environmental and physiological factors influencing these traits.

The PCA results highlight the metabolic changes that occur in kale accessions after frost and reveal the complex biochemical adaptations to abiotic stress. This metabolic segregation along Dim1 reflects a frost-triggered reconfiguration of central metabolic networks, involving both the modulation of glucosinolate biosynthesis and alterations in photosynthetic pigment pathways, suggesting stress-driven regulation at the transcriptional and post-transcriptional levels. This result is in line with previous studies, such as those by Gantait et al. (2024), who identified glucosinolates and photosynthetic variables as important metabolic markers for abiotic stress responses in *Brassica* species. The metabolic profile of pre-frost kale accessions, characterized by higher levels of aliphatic glucosinolates and carbohydrate-related metabolites, reflects their involvement in supporting normal metabolic activity and energy equilibrium under non-stress conditions. Similar transitional metabolic states have been reported in Brassicaceae, where certain accessions exhibit signs of metabolic preparedness or partial pre-conditioning before stress exposure (Ben Ammar, 2025; Itabashi et al., 2018; Zhu et al., 2016). The metabolic profile of Accession_27 occupies an intermediate position between the two treatment groups, suggesting that it may deviate from the canonical stress-response pattern observed in the other accessions. While this study was not designed to directly test such a mechanism, this metabolic positioning may indicate an accession-specific regulatory configuration that becomes active before environmental stress is encountered. To evaluate this hypothesis, future investigations could combine metabolomic profiling with genomic and regulatory analyses. Targeted approaches, including stress-recovery assays, transcript profiling, methylation analysis, and chromatin-accessibility mapping (e.g., ATAC-seq) may help determine whether the metabolic features of Accession_27 are genetically encoded or reflect regulatory flexibility consistent with stress-priming phenomena. Clarifying this distinction would provide valuable insights into how genotype and regulatory mode jointly shape adaptive metabolic responses in Brassicaceae. The proximity of Unknown 2 to aliphatic glucosinolates and carbohydrate-related metabolites in the pre-frost group indicates a potential association with metabolic stabilisation and energy maintenance under optimal conditions. Given the importance of glucosinolates in plant secondary metabolism (Jeschke et al., 2019), it is plausible that plants modulate their glucosinolate biosynthesis to support metabolic adaptation and response to stress. Future work could investigate the role of Unknown 2 in these processes. On the other hand, the accessions under frost stress show a clear association with aromatic and indolic glucosinolates, which are involved in cold tolerance mechanisms in frost-adapted plants (Jurkow et al., 2019). Kale Accession_8 exhibited a distinct metabolic signature under frost conditions, characterised by elevated levels of several stress-related metabolites. This may indicate a potential adaptive response; however, additional physiological validation is required to confirm cold tolerance. This accession could be a valuable target for breeding programmes aimed at improving the frost resistance of kale. The involvement of unidentified glucosinolates, especially Unknown 1 and Unknown 3, in frost stress pathways is a fascinating finding. Their grouping with aromatic and indolic glucosinolates suggests that these uncharacterised metabolites may play a crucial role in the plant's response to frost. This aligns with the hypotheses of Zhu et al. (2024), who investigated uncharacterised secondary metabolites involved in stress adaptation. The identification and

characterisation of these metabolites could provide new insights into the mechanisms of cold stress responses and contribute to a deeper understanding of plant resilience under environmental stress. In contrast to previous studies conducted under controlled laboratory conditions, the experiment was conducted in open-field, allowing a more realistic assessment of the effects of low temperatures on the physiological status and phytochemical composition of kale. The field-based experimental design captures the cumulative impact of uncontrolled environmental interactions, including soil heterogeneity, natural light variability, humidity and temperature fluctuations that are typically omitted in controlled experiments. While these factors were not modelled explicitly, they are inherently embedded within the residual variance, thus reflecting the ecological complexity of real agronomic systems. By exposing the plants to real climate fluctuations, this study provides more ecologically relevant insights into how temperature fluctuations affect nutrient accumulation. The results thus provide valuable guidance for agricultural practise, particularly for optimising kale production under cold stress conditions.

5. Conclusion

This study highlights the metabolic reprogramming of kale varieties in response to frost stress and emphasise the role of sugar metabolism and glucosinolate dynamics in cold tolerance. The significant accumulation of sucrose before frost indicates its function as an osmoprotectant. Remarkably, Accession_4 showed the highest sucrose accumulation, suggesting strong osmoprotective potential and making it a promising candidate for cold-tolerance breeding strategies. In contrast, Accession_8 showed a distinct metabolic signature, but its position in the PCA space indicates that this variation is accession-specific rather than clearly associated with frost adaptation. Further investigation is needed to determine whether its metabolic behaviour represents an alternative stress-response strategy or inherent genotype variability. The observed differences between kale accessions emphasise the importance of genetic diversity in metabolic responses to environmental stresses. In addition, the reduction in chlorophyll and carotenoid content indicate a possible impairment of photosynthetic efficiency after frost, which exacerbates the effects of cold stress on plant physiological performance. These results provide valuable insights for breeding programmes aimed at improving the resistance of kale to cold stress while optimising its nutritional and bioactive compound profiles. Future studies should further investigate the genetic basis of these metabolic adaptations to facilitate the development of cold-tolerant kale varieties.

CRediT authorship contribution statement

Lovro Sinkovič: Writing – original draft, Methodology, Formal analysis, Conceptualization. **Barbara Pipan:** Writing – review & editing, Resources, Methodology, Data curation, Conceptualization. **Mohamed Neji:** Writing – original draft, Software, Methodology, Conceptualization. **Hajer Ben Ammar:** Writing – original draft, Methodology, Conceptualization. **Vladimir Meglič:** Writing – review & editing, Funding acquisition. **Robert Veberič:** Writing – review & editing, Supervision, Conceptualization. **Ana Slatnar:** Writing – review & editing, Visualization, Conceptualization. **Jerneja Jakopič:** Writing – review & editing, Methodology, Formal analysis, Data curation.

Declaration of competing interest

The authors mentioned above declare that they have no conflict of interest.

Acknowledgments

The authors would like to express their gratitude to Živa Uranič and Ana Vojnović for their invaluable assistance and technical support in the laboratory analysis. This study was conducted as part of the research programme Agrobiodiversity (P4-0072) and Horticulture (P4-0013) financially supported by the Slovenian Research and Innovation Agency (ARIS), Ljubljana, Slovenia; part of the EUBRASWILD project funded by the international ECPGR project (Third Call – Phase X 2019–2023); and part of the bilateral projects funded by ARIS: Slovenia-Serbia (BI-RS/20-21-015) and Slovenia-Bosnia and Herzegovina (BI-BA/19-20-009 and BI-BA/21-23-011).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jplph.2026.154695.

Data availability

Data will be made available on request.

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