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SC: supervised the research and designed the experiment; RJ, AW: performed field study and laboratory analyses; RJ: analyzed the data and wrote the manuscript with support from AK; AK, AS, SC: provided critical feedback and revised the manuscript

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**Competing interests**

No competing interests have been declared.

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## ORIGINAL RESEARCH PAPER

# Cold stress modifies bioactive compounds of kale cultivars during fall–winter harvests

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**Abstract**

Kale is a plant known and valued since antiquity as a healthy vegetable crop, used for culinary, decorative, but also healing purposes. The aim of the study was to examine the effect of harvest date on physiological status and nutritional composition of two kale cultivars: 'Winterbor' F<sub>1</sub> (blue-green leaves) and 'Redbor' F<sub>1</sub> (red-purple leaves). The leaves were harvested in three periods: before frost (>0°C), after medium (–5.0°C) and heavy frost (–15.0°C). Content of dry weight, soluble sugars, L-ascorbic acid, carotenoids, chlorophylls, polyphenols, anthocyanins, as well as antioxidant activity and peroxidase activity were determined. Cold temperature significantly affected bioactive compounds of kale. The content of dry weight, soluble sugars, L-ascorbic acid, phenolics, and antioxidant activity increased after medium frosts for both cultivars. The level of anthocyanins also increased significantly for the 'Redbor' F<sub>1</sub> cultivar. After strong frost, most of the tested parameters (content of dry weight, soluble sugars, phenolics, anthocyanins, and total antioxidant and peroxidase activity) significantly increased. The chlorophyll *a* content was reduced by heavy frost in both seasons. Harvesting kale before and after frost may allow the level of biologically active ingredients to be regulated as cold also significantly affects the physiological status of the plants.

**Keywords**

*Brassica oleracea* L. var. *acephala*; harvest date; cold stress; nutritional value

**Introduction**

Kale (*Brassica oleracea* L. var. *acephala*) is the one of the oldest forms of the Brassicaceae family, native to the eastern Mediterranean. It is a plant with edible, strongly curled leaves of green to dark-red color. Leaves are characterized by the highest nutritional value among *Brassica* vegetables [1]. This crop is cultivated mainly for leaves, while the flowers, appearing in early spring, can also enrich the diet, because they have high nutritional value [2,3]. Kale has a specific taste, reminiscent of cabbage. Under field conditions, after frost, the taste of kale leaves becomes sweeter and more delicate. The loss of bitterness is characteristic for plants from the Brassicaceae family. Frequent consumption of fresh or frozen leaves enriches the diet and has a positive effect on human health attributed to their large amount of constituents with strong antioxidant capacity [4]. The cultivation of kale in the world has been known since antiquity. Due to low optimum growing temperature, kale is usually cultivated mostly in the cold season (late summer to December–January) at low latitudes, and in the summer at higher latitudes [5]. The greatest advantages of kale are low climate and soil requirements and easy cultivation in comparison to other *Brassica* crops. Kale is one of a few vegetables available fresh on the market throughout the winter. Recently, many studies have focused on the nutritious composition of kale [5,6]. Kale has the highest nutritional value according to the ANDI scale (aggregate nutrient density index) [7]. Its leaves are rich in nutrients and bioactive compounds: proteins, fiber, carotenoids,

vitamin C, vitamins of B group, as well as other vitamins such as: A, PP, E, K, and H. Kale, compared to other vegetables of the Brassicaceae family, contains most vitamin C (120 mg 100 g<sup>-1</sup>), vitamin A (892 µg 100 g<sup>-1</sup>), β-carotene (5,350 µg 100 g<sup>-1</sup>), vitamins B<sub>1</sub> (equal with cauliflower: 0.11 mg 100 g<sup>-1</sup>) and PP (1.6 mg 100 g<sup>-1</sup>), potassium (530 mg 100 g<sup>-1</sup>), calcium (157 mg 100 g<sup>-1</sup>), iron (1.7 mg 100 g<sup>-1</sup>) (all values are given per 100 g of fresh weight) [8]. This vegetable crop is a particularly rich source of vitamin C together with sweet pepper and parsley.

Kale is the most resistant to low temperature among the *Brassica* vegetables [9]. It can grow very well during cold months, at a temperature of a few degrees above zero. The plant can survive temperatures up to -20°C. However, low temperature or cold stress is a factor that can affect the growth and productivity of plants and also lead to significant yield losses [10]. Low temperature influences many physiological changes and biochemical pathways in plants [11], mainly by inducing production of ROS (reactive oxygen species), which modify membrane fatty acid composition and change gene expression to increase production of radical scavengers as well as osmoprotectants maintaining homeostasis [12].

Under conditions of low temperature, an increase is observed in the content of nonenzymatic antioxidant compounds and activity of enzymatic antioxidants that play a role as radical scavengers removing ROS [13]. The most important nonenzymatic antioxidants include such compounds as: glutathione, ascorbic acid, flavonoids, tocopherols, or carotenoids [14]. Kalisz et al. [15] observed that chilling increased L-ascorbic acid content in most tested basil cultivars. Low temperature may also affect the content of phenolics and anthocyanins in the plant [14,16]. Lee and Oh [17] described an increase in total phenolics concentration in kale plants subjected to low temperature conditions. Steyn et al. [18] showed that plants can accumulate anthocyanins as a result of various environmental factors that cause abiotic stress. Anthocyanins can also act as osmoregulators in cells. Due to the fact that they are osmotically active, their accumulation in vacuoles may allow adequate water potential to be maintained in conditions of water deficit and the crystallization of ice to be prevented [19]. Changes that occur in soluble sugar levels due to cold stress are most often associated with freezing tolerance. Soluble sugars are important factors that contribute to cold acclimation as low-molecular-weight osmolytes accumulating in frost-resistant plant cells. During a long exposition of plants in the field to low temperatures, the anabolic processes are inhibited [20]. As a result, the consumption of sugar is minimized, but parallel sugar production occurs during sunlight [21]. The accumulation of these compounds may also affect the flavor of kale, resulting in increased sweetness and delicacy of harvested leaves [5]. D'Antuono and Neri [22] recommended to delay the harvest of kale during fall in order to obtain high quality of leaves. The activity of antioxidant enzymes, including superoxide dismutase, peroxidase, and catalase, usually increases in plants in stressful conditions [23]. Almughraby et al. [24] showed that short-term frost (-5°C) led to an increase in the activity of enzymes regulating ROS content in plant cells. Such activation can be associated with induced synthesis of antioxidant enzymes [25]. Peroxidase plays an important role in plants by the regulation of several life processes. Activity of peroxidase may increase when the plants are subjected to pathogen or abiotic stress [26,27]. Biczak et al. [28] confirmed that changes in the activity of antioxidant enzymes, like peroxidase, may indicate the presence of oxidative stress in plants.

The aim of this study was to examine the impact of three harvesting dates on the biological value of two kale cultivars: 'Winterbor' F<sub>1</sub> (blue-green leaves) and 'Redbor' F<sub>1</sub> (red-leaved plant). Kale is a typical plant for fall and winter crops. Thus, this research was to determine how the biological value may change depending on low temperatures occurring in open field during the winter season.

## Material and methods

### Experimental conditions

Transplant production was carried out in the greenhouse of the University of Agriculture in Krakow, Poland. Seeds of two kale cultivars: 'Winterbor' F<sub>1</sub> (blue-green leaves) and

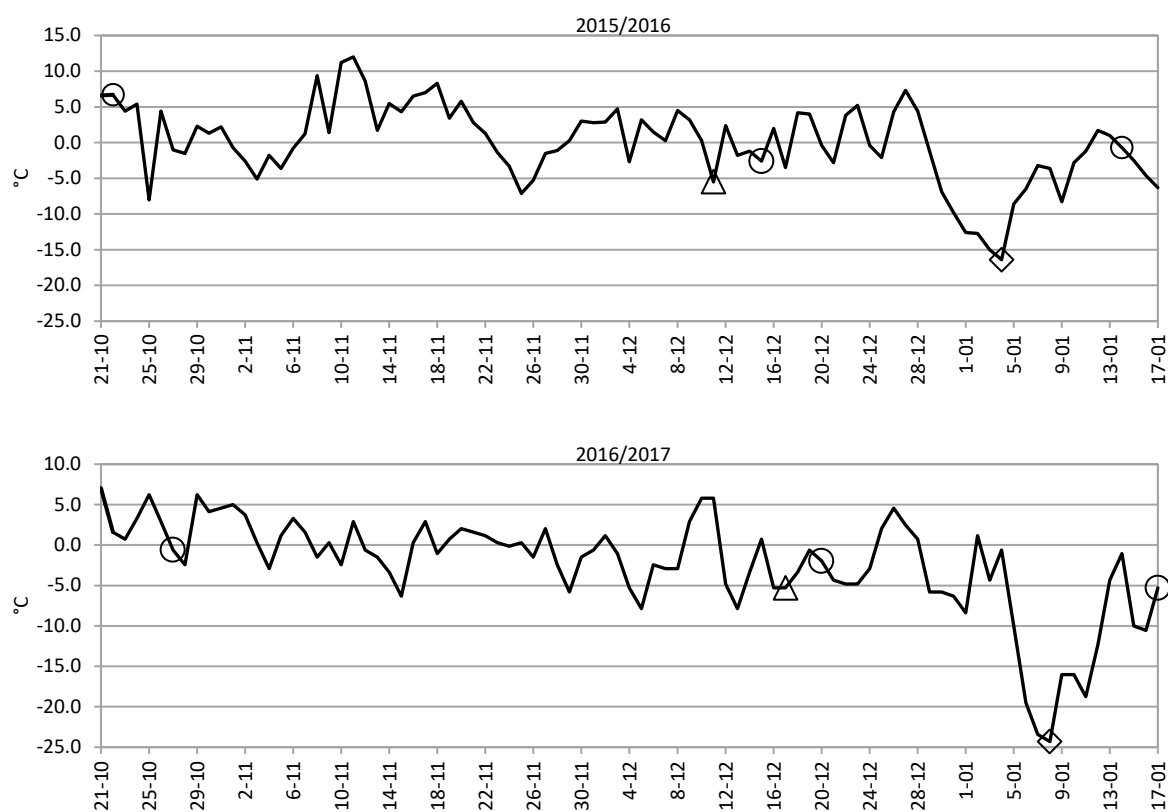
'Redbor' F<sub>1</sub> (red-purple leaves) (Bejo Zaden Poland), were seeded into multicell trays with 96 cells (VEFI A/S, Norway), filled with peat substrate (Klasman TS2; Klasmann-Deilmann GmbH, Germany); the volume of a single cell was 53 cm<sup>3</sup>. Sowing was performed on May 28, 2015 and May 30, 2016. Thermal conditions that prevailed in the greenhouse were about 23°C ±2°C in daytime, while at night it was 19°C ±2°C. The field experiment was carried out in the experimental station of the University of Agriculture in Krakow (50°04' N, 19°5' E). Soil is classified as Fluvisol Cambisol (Humic) according to the classification of the Food and Agriculture Organization of the United Nations. According to the Köppen classification, the climate of the region is humid continental (Dfb).

### Experimental design

The experiment was based on the two-factorial method, comparing two kale cultivars and three harvest dates: I – before frost (>0°C); II – after medium frost (–5°C); III – after heavy frost (–15°C). Harvest II was performed usually 5 days after medium frost, and Harvest III – after next 10 days after heavy frost so that there was enough time for plants to change metabolic pathways and in consequence their chemical composition. The experimental design used randomized sub-blocks in four replicates. Each experimental plot consisted of 30 plants (10.1 m<sup>2</sup>), surrounded by shelterbelts. Sowing was performed on May 28, 2015 and May 30, 2016. Transplants were planted to the field on June 25, 2015 and June 23, 2016 at a spacing of 67.5 × 50 cm. In 2015, the first harvest was carried out on October 22 (120 days after transplanting – DAT), the second one on December 15, 2015 (DAT = 174; after the temperature dropped to –5.5°C), while the last harvest was performed on January 14 (DAT = 204; after the temperature dropped to –16.4°C). In 2016, the first harvest of kale leaves was performed on October 27 (DAT = 126), the next harvest took place after medium frost (up to –7°C) on December 20 (DAT = 180), and the last one on January 17 (DAT = 208), after strong frost, when the temperature dropped to almost –24°C. The beginning and mid-January were snowy, and snow remained on the plants until the day of the last harvest. Tillage, irrigation, fertilization, weed management, and plant protection were conducted in agreement with the requirements of common horticultural recommendations for kale.

### Microclimatic conditions

Daily reports on temperatures and rainfall were obtained from the Krakow-Balice meteorological station, located close to the experimental field. In 2015, the first harvest was carried out when there was no temperature drop below 0°C. Before the next harvesting date, the lowest temperature of –8.0°C, –7.1°C, and –5.5°C was recorded. The lowest temperature drop was observed at the turn of December and January. Overall, the lowest temperature of –16.4°C was registered on January 4, 2016. In 2016, the field experiment was carried out under similar microclimatic conditions. In the period between the first and last harvest, the daily temperatures dropped below 0°C more often in comparison to the previous season. A few clearly warmer days were observed on November 18 and 21, when the largest daily amplitude occurred, on December 10 and 26, as well as on January 13. However, frost that occurred on December 5 and 13 was a key for the experiment. Therefore, it was possible to proceed to the next harvest of kale leaves a few days later. The lowest daily air temperature took place on January 8, when it reached less than –24.3°C. Then, it gradually increased so that on the day of the last harvest it was –5.3°C. Between the first and last harvest, 22 days were observed with precipitation. The highest daily precipitation occurred on December 2, 12, and 25. The pattern of minimum air temperature in the fall and winter season in the 2015/2016 and 2016/2017 is presented in Fig. 1.



**Fig. 1** Minimum air temperatures during fall–winter of 2015/2016 and 2016/2017 seasons. Circles indicate the dates of harvesting kale plants (from the left: I, II, and III, respectively); triangles – medium frost; diamonds – heavy frost.

#### Plant sampling and laboratory analysis

One leaf was cut from the central part from the west side of the plant, sampling it from eight plants per treatment. Leaves were sampled in each consecutive harvest from different randomly selected plants. They were immediately transported to the laboratory where analyses were carried out. Only leaf blades were used as plant material for further analyses.

Determination of dry weight was performed by the Pijanowski drying method [24]. The fragmented plant material was weighed, using a Sartorius A120S analytical balance (Sartorius AG, Germany), and dried at 65°C. After drying, the samples were weighed again and the results were converted into the percentage of dry weight in fresh weight [29].

Soluble sugars content was determined by the anthrone (colorimetric) method. The plant material was mixed with 80% ethanol. Following addition of anthrone, the samples were placed in a water bath for 30 min (100°C) and cooled down to 20–22°C. The sugars contained in the sample merged with the anthrone reagent to form a green-blue color whose intensity was measured in a UV-VIS Helios Beta spectrophotometer (Thermo Fisher Scientific, Inc., USA) at a wavelength of 625 nm. The intensity of the obtained color is proportional to the content of sugars in the tested plant sample. The total soluble sugars content was calculated using values read from the standard curve of glucose [30].

Determination of L-ascorbic acid content was performed by the Tillmans method [31]. This method involves the extraction of L-ascorbic acid with oxalic acid and subsequent oxidation in an acidic environment of L-ascorbic acid to dehydroxy-ascorbic acid with 2,6-dichlorophenindophenol. Excessive dye in an acidic environment gives a pink color and marks the end point of the titration. The content of L-ascorbic acid in the sample was calculated based on the amount of the changed solution of 2,6-dichlorophenindophenol used for titration.

Contents of carotenoids and chlorophylls were evaluated according to the Lichenthaler and Wellburn method. The pigments were extracted in 80% (v/v) aqueous

acetone (25 cm<sup>3</sup>). After 0.5 h in the dark, absorbance of the extracts was read at 663, 646, and 470 nm on a UV-VIS Helios Beta spectrophotometer. Then, the content of chlorophyll *a*, chlorophyll *b*, and carotenoids was calculated from the formula described by Lichtenthaler and Wellburn [32].

Phenolic compounds were determined according to the Folin–Ciocalteu method. They were extracted from plant tissues with an 80% methanol solution. The basis for the determination was the reversible reduction reaction by phenols in the alkaline environment of molybdenum (VI) to molybdenum (V) contained in the Folin–Ciocalteu (F–C) reagent. The reaction resulted in a blue compound that exhibits a maximum absorption at 745–750 nm. The absorption intensity at this wavelength is proportional to the concentration of phenolic compounds in the plant sample [33].

Anthocyanins were extracted from the plant material with a solution of concentrated ethanol and concentrated hydrochloric acid, mixed in a ratio of 85:15 by volume. The absorbance of the obtained solutions was measured at 520 nm, after mixing the extract with buffer at pH = 1 (1:4; v/v) and buffer at pH = 4.5 (1:4; v/v). In addition, the absorbance of the obtained solutions at a wavelength of 700 nm was measured to correct the results by the turbidity of the solution. The content of anthocyanins was converted to cyanidin-3-glucoside, using a molar absorbance factor  $\epsilon = 26,900 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$  [34].

Antioxidant activity was determined using the DPPH<sup>•</sup> radical (2,2-diphenyl-1-picrylhydrazyl). The method uses the reduction of the DPPH<sup>•</sup> radical by antioxidants contained in the plant material. The radical solution changed the violet color to yellow, and the decrease in absorbance over time was measured in a UV-VIS Helios Beta spectrophotometer at 517 nm, relative to the reference solution. Antioxidative activity was expressed as a percentage of residual DPPH<sup>•</sup> not reduced by the antioxidants contained in the sample. In this method, the amount of residual DPPH<sup>•</sup> radical is inversely proportional to the content of antioxidants in the plant material [35].

Determination of peroxidase activity was performed according to the Lück method [36]. Peroxidase activity was determined using *p*-phenylenediamine and H<sub>2</sub>O<sub>2</sub> as the enzyme substrate. The absorbance was measured at 60-second intervals up to 2 minutes in a UV-VIS Helios Beta spectrophotometer at 485 nm. Enzymatic activity is expressed as U units, where 1 U is the increase in absorbance by 0.1 per minute per 1 g of plant tissue.

### Statistical analysis

Statistical analyses were performed in STATISTICA 12.0 (StatSoft, Inc., USA). A two-way analysis of variance (ANOVA) was used to determine the main effects of harvest date and kale genotype as well as interactions among main effects. Anthocyanins content was analyzed by one-way ANOVA taking into account harvest date as an experimental factor. Homogenous groups were separated with Tukey's HSD test, with a significance level of  $p \leq 0.05$ .

### Results

Due to ever-lower temperatures, the kale cultivars showed an increase in dry weight content in their tissues and its level reached maximum after the occurrence of strong frost in both seasons (Tab. 1, Tab. 2). The means for the main effect (harvest date) confirmed such dependence. In both seasons, 'Redbor' F<sub>1</sub> was characterized by a higher amount of dry weight. The dry weight content increased with the following harvesting dates. At Harvest Date III in the 2015/2016 season, the mean value for the cultivars increased to 25.02% FW. In the 2016/2017 season, this value increased to 28.04% FW.

In the present experiment, the content of soluble sugars increased with the harvest date in both seasons (Tab. 1, Tab. 2). During the third harvest (after heavy frost), the average values were 4.53% FW and 6.00% FW for the successive seasons. Before frost, these values reached 2.72% FW and 2.43% FW in first and second season, respectively. A higher amount of sugars was found in 'Winterbor' F<sub>1</sub> in comparison to 'Redbor' F<sub>1</sub>.

**Tab. 1** Content of dry weight, soluble sugars, and L-ascorbic acid in two kale cultivars in 2015/2016 season.

Kale cultivar	Harvest date	Dry weight (% FW)	Soluble sugars (% FW)	L-Ascorbic acid (mg 100 g <sup>-1</sup> FW)
'Winterbor'	I	16.67 <sup>a</sup>	2.68 <sup>a</sup>	115.65 <sup>a</sup>
	II	20.34 <sup>c</sup>	4.37 <sup>c</sup>	148.01 <sup>c</sup>
	III	21.33 <sup>d</sup>	4.28 <sup>c</sup>	132.18 <sup>b</sup>
'Redbor'	I	19.29 <sup>b</sup>	2.75 <sup>a</sup>	146.64 <sup>c</sup>
	II	21.58 <sup>d</sup>	3.55 <sup>b</sup>	152.08 <sup>d</sup>
	III	28.71 <sup>e</sup>	4.78 <sup>d</sup>	147.51 <sup>c</sup>
<b>Means for harvest date (H)</b>				
I		17.98 <sup>A</sup>	2.72 <sup>A</sup>	131.15 <sup>A</sup>
II		20.96 <sup>B</sup>	3.96 <sup>B</sup>	150.05 <sup>C</sup>
III		25.02 <sup>C</sup>	4.53 <sup>C</sup>	139.85 <sup>B</sup>
<b>Means for cultivar (C)</b>				
'Winterbor'		19.45 <sup>A</sup>	3.78 <sup>B</sup>	131.95 <sup>A</sup>
'Redbor'		23.19 <sup>B</sup>	3.69 <sup>A</sup>	148.74 <sup>B</sup>

Means within a column followed by different letters (capital letters for main effect of harvest date H and cultivar C; lowercase letters for interaction effect H × C) are significantly different at  $p \leq 0.05$ , with comparisons performed using Tukey's HSD test,  $n = 4$ . Harvest date: I – before frost; II – after medium frost; III – after heavy frost.

**Tab. 2** Content of dry weight, soluble sugars, and L-ascorbic acid in two kale cultivars in 2016/2017 season.

Kale cultivar	Harvest date	Dry weight (% FW)	Soluble sugars (% FW)	L-Ascorbic acid (mg 100 g <sup>-1</sup> FW)
'Winterbor'	I	14.01 <sup>a</sup>	2.53 <sup>a</sup>	114.34 <sup>ab</sup>
	II	21.23 <sup>c</sup>	3.87 <sup>b</sup>	169.49 <sup>c</sup>
	III	27.32 <sup>d</sup>	6.94 <sup>d</sup>	133.14 <sup>b</sup>
'Redbor'	I	17.09 <sup>b</sup>	2.32 <sup>a</sup>	120.17 <sup>b</sup>
	II	23.15 <sup>c</sup>	3.72 <sup>b</sup>	196.40 <sup>d</sup>
	III	28.76 <sup>d</sup>	5.05 <sup>c</sup>	91.22 <sup>a</sup>
<b>Means for harvest date (H)</b>				
I		15.55 <sup>A</sup>	2.43 <sup>A</sup>	117.26 <sup>A</sup>
II		22.19 <sup>B</sup>	3.80 <sup>B</sup>	182.95 <sup>B</sup>
III		28.04 <sup>C</sup>	6.00 <sup>C</sup>	112.18 <sup>A</sup>
<b>Means for cultivar (C)</b>				
'Winterbor'		20.85 <sup>A</sup>	4.45 <sup>B</sup>	138.99 <sup>A</sup>
'Redbor'		23.00 <sup>B</sup>	3.70 <sup>A</sup>	135.93 <sup>A</sup>

For notes, see [Tab. 1](#).

The highest amount of L-ascorbic acid was observed during the second harvest ([Tab. 1](#), [Tab. 2](#)). The average content of this compound in 2015/2016 was 150.05 mg 100 g<sup>-1</sup> FW, and in the next season: 182.95 mg 100 g<sup>-1</sup> FW. The initial content of vitamin C was lower by 18.90 and 65.69 mg 100 g<sup>-1</sup> FW in comparison to the next harvest in the first and second season, respectively, while during the last harvest, after heavy frost, it dropped by 10.20 and 70.77 mg 100 g<sup>-1</sup> FW, as compared to Harvest Date II.

**Tab. 3** Content of chlorophyll *a*, chlorophyll *b*, chlorophyll *a+b*, and carotenoids in two kale cultivars in 2015/2016 season.

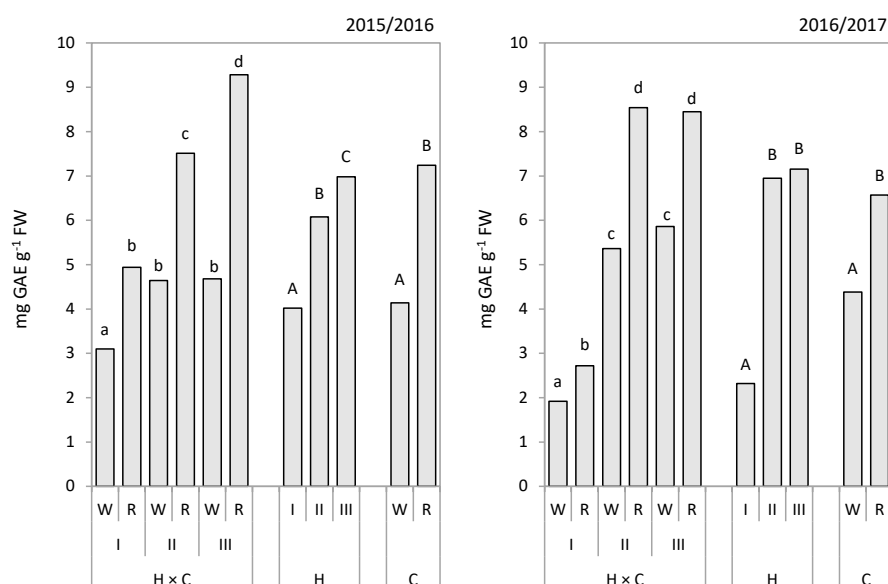
Kale cultivar	Harvest date	Chlorophyll <i>a</i> (mg g <sup>-1</sup> FW)	Chlorophyll <i>b</i> (mg g <sup>-1</sup> FW)	Chlorophyll <i>a+b</i> (mg g <sup>-1</sup> FW)	Carotenoids (mg g <sup>-1</sup> FW)
'Winterbor'	I	1.240 <sup>bc</sup>	0.632 <sup>a</sup>	1.872 <sup>ab</sup>	0.142 <sup>a</sup>
	II	1.238 <sup>bc</sup>	0.651 <sup>a</sup>	1.889 <sup>ab</sup>	0.221 <sup>bc</sup>
	III	1.010 <sup>a</sup>	0.602 <sup>a</sup>	1.612 <sup>a</sup>	0.170 <sup>ab</sup>
'Redbor'	I	1.348 <sup>c</sup>	0.758 <sup>ab</sup>	2.106 <sup>bc</sup>	0.242 <sup>cd</sup>
	II	1.188 <sup>b</sup>	0.741 <sup>ab</sup>	1.929 <sup>bc</sup>	0.296 <sup>e</sup>
	III	1.361 <sup>c</sup>	0.821 <sup>b</sup>	2.182 <sup>c</sup>	0.293 <sup>de</sup>
<b>Means for harvest date (H)</b>					
I		1.294 <sup>B</sup>	0.695 <sup>A</sup>	1.989 <sup>A</sup>	0.192 <sup>A</sup>
II		1.213 <sup>A</sup>	0.696 <sup>A</sup>	1.909 <sup>A</sup>	0.259 <sup>B</sup>
III		1.186 <sup>A</sup>	0.712 <sup>A</sup>	1.897 <sup>A</sup>	0.232 <sup>B</sup>
<b>Means for cultivar (C)</b>					
'Winterbor'		1.163 <sup>A</sup>	0.628 <sup>A</sup>	1.791 <sup>A</sup>	0.178 <sup>A</sup>
'Redbor'		1.299 <sup>B</sup>	0.773 <sup>B</sup>	2.072 <sup>B</sup>	0.277 <sup>B</sup>

For notes, see [Tab. 1](#).**Tab. 4** Content of chlorophyll *a*, chlorophyll *b*, chlorophyll *a+b*, and carotenoids in two kale cultivars in 2016/2017 season.

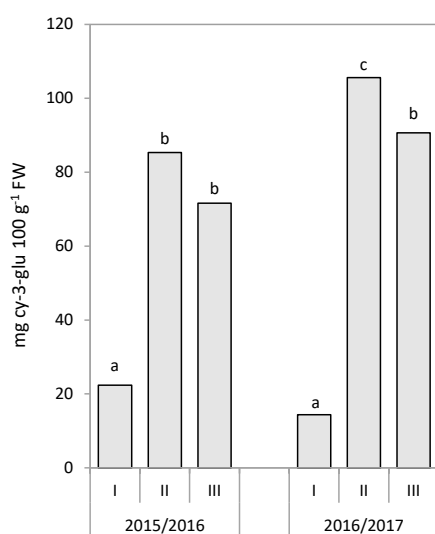
Kale cultivar	Harvest date	Chlorophyll <i>a</i> (mg g <sup>-1</sup> FW)	Chlorophyll <i>b</i> (mg g <sup>-1</sup> FW)	Chlorophyll <i>a+b</i> (mg g <sup>-1</sup> FW)	Carotenoids (mg g <sup>-1</sup> FW)
'Winterbor'	I	1.531 <sup>b</sup>	0.490 <sup>ab</sup>	2.021 <sup>bcd</sup>	0.324 <sup>a</sup>
	II	1.416 <sup>ab</sup>	0.452 <sup>ab</sup>	1.868 <sup>abc</sup>	0.331 <sup>a</sup>
	III	1.235 <sup>a</sup>	0.398 <sup>a</sup>	1.633 <sup>a</sup>	0.322 <sup>a</sup>
'Redbor'	I	1.616 <sup>b</sup>	0.590 <sup>cd</sup>	2.206 <sup>d</sup>	0.414 <sup>b</sup>
	II	1.493 <sup>b</sup>	0.625 <sup>d</sup>	2.118 <sup>cd</sup>	0.455 <sup>b</sup>
	III	1.226 <sup>a</sup>	0.514 <sup>bc</sup>	1.740 <sup>ab</sup>	0.353 <sup>a</sup>
<b>Means for harvest date (H)</b>					
I		1.574 <sup>B</sup>	0.540 <sup>B</sup>	2.114 <sup>B</sup>	0.369 <sup>B</sup>
II		1.455 <sup>B</sup>	0.539 <sup>B</sup>	1.993 <sup>B</sup>	0.393 <sup>B</sup>
III		1.231 <sup>A</sup>	0.456 <sup>A</sup>	1.687 <sup>A</sup>	0.338 <sup>A</sup>
<b>Means for cultivar (C)</b>					
'Winterbor'		1.394 <sup>A</sup>	0.447 <sup>A</sup>	1.841 <sup>A</sup>	0.326 <sup>A</sup>
'Redbor'		1.445 <sup>A</sup>	0.576 <sup>B</sup>	2.021 <sup>B</sup>	0.407 <sup>B</sup>

For notes, see [Tab. 1](#).

The chlorophyll *a* content was the highest at the first harvest in the 2015/2016 season ([Tab. 3](#)). No effect of harvest date on chlorophyll *b* and total chlorophyll content was observed at that time. However, in the season 2016/2017 heavy frost decreased the content of chlorophyll *a*, chlorophyll *b*, and, in consequence, the total chlorophylls, which was reflected in the data of the last sampling ([Tab. 4](#)). The average content of chlorophyll *a* for 'Winterbor' F<sub>1</sub> was 1.163–1.394 mg g<sup>-1</sup> FW in the successive seasons, for 'Redbor' F<sub>1</sub> it was 1.299–1.445 mg g<sup>-1</sup> FW. In the season 2016/2017, differences between cultivars in chlorophyll *a* content were insignificant. 'Redbor' F<sub>1</sub> contained significantly more chlorophyll *b* (0.773 and 0.576 mg g<sup>-1</sup> FW in the first and second season, respectively,



**Fig. 2** Total phenolics content in two kale cultivars (W – ‘Winterbor’ F<sub>1</sub>; R – ‘Redbor’ F<sub>1</sub>) as affected by harvest date (I – before frost; II – after medium frost; III – after heavy frost) in the seasons 2015/2016 and 2016/2017. Means followed by different letters (capital letters for main effect of harvest date H and cultivar C; lowercase letters for interaction effect H × C) are significantly different at  $p \leq 0.05$  according to Tukey’s HSD test.



**Fig. 3** Anthocyanins content in ‘Redbor’ F<sub>1</sub> cultivar as affected by harvest date (I – before frost; II – after medium frost; III – after heavy frost) in the seasons 2015/2016 and 2016/2017. Means, separately for seasons, followed by different letters are significantly different at  $p \leq 0.05$  according to Tukey’s HSD test.

than ‘Winterbor’ F<sub>1</sub> (0.629–0.447 mg g<sup>-1</sup> FW). Similar dependencies were also observed for the total chlorophyll content.

The content of carotenoids increased only in the 2015/2016 season after the second and third harvest (Tab. 3, Tab. 4). After these harvests, kale had a higher content of carotenoids by 0.066–0.040 mg g<sup>-1</sup> FW in comparison to the initial value. It was also shown that a higher amount of carotenoids was found in ‘Redbor’ F<sub>1</sub> (0.277–0.407 mg g<sup>-1</sup> FW, on average), while ‘Winterbor’ F<sub>1</sub> had only 0.178–0.325 mg g<sup>-1</sup> FW.

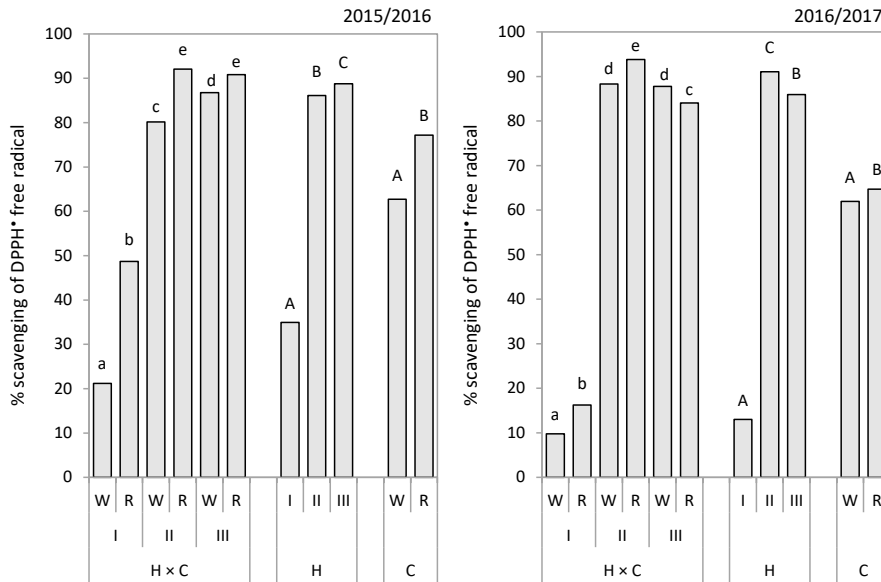
The content of phenolics was the lowest in both kale cultivars at Harvest Date I (Fig. 1, Fig. 2). Medium frost significantly increased phenolics content in both seasons. After heavy frost, the level of total phenolics increased in the tested cultivars, with an exception of ‘Winterbor’ F<sub>1</sub> in 2015/2016. There was no difference in phenolics content between Harvest Dates I and II in the 2016/2017 season. ‘Redbor’ F<sub>1</sub> contained 6.57–7.24 mg GAE g<sup>-1</sup> FW; it was almost twice as high as in ‘Winterbor’ F<sub>1</sub> (4.14–4.38 mg GAE g<sup>-1</sup> FW).

Anthocyanins, determined only for the ‘Redbor’ F<sub>1</sub> cultivar, showed similar dependencies as total phenolics content. Both medium and heavy frost increased the concentration of anthocyanins in kale leaves, but heavy frost decreased their content in comparison to medium frost in the 2016/2017 season (Fig. 3), but this difference was not observed for total phenolics at that time. After medium frost, the level of anthocyanins was 85.32 mg 100 g<sup>-1</sup> FW in the 2015/2016 season, and then this level decreased to 71.61 mg 100 g<sup>-1</sup> FW. In the 2016/2017 season, the largest concentration of anthocyanins was observed during the second harvest (105.62 mg 100 g<sup>-1</sup> FW).

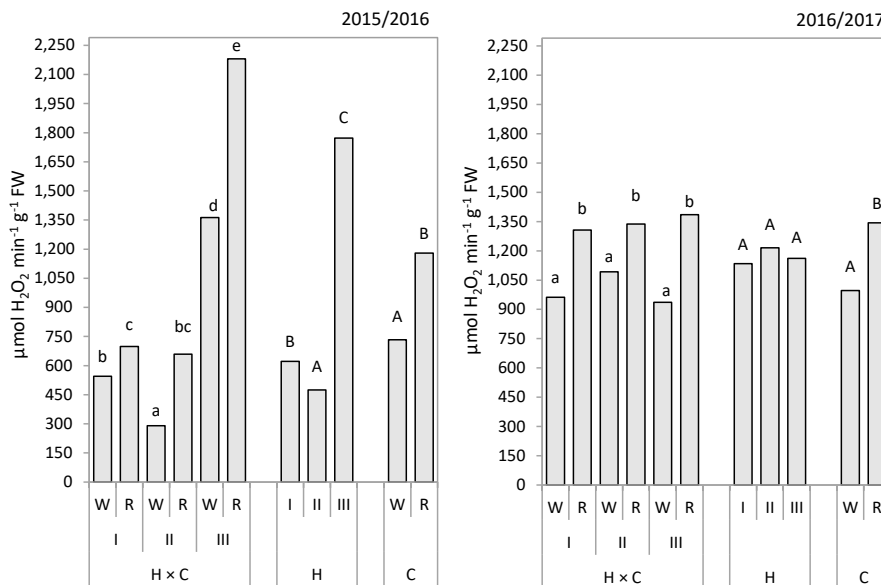
Total antioxidant activity increased significantly after frost (Fig. 4). At the first harvest, in the 2015/2016 season, it was 34.94% of the ability of DPPH<sup>•</sup> free radical scavenging, while in 2016/2017 only 13.01%. After medium frost, these values increased up to 86.09% and 91.07%, respectively, for the successive seasons. Heavy frost increased scavenging of DPPH<sup>•</sup> radicals in 2015/2016, while in the next season this activity was lower after heavy frost than after medium frost.

In the present study, an increase in peroxidase activity after heavy frost, but not medium frost, was observed in the season 2015/2016 (Fig. 5). In the next season, no





**Fig. 4** DPPH\* radical scavenging activity in two kale cultivars (W – ‘Winterbor’ F<sub>1</sub>; R – ‘Redbor’ F<sub>1</sub>) as affected by harvest date (I – before frost; II – after medium frost; III – after heavy frost) in the seasons 2015/2016 and 2016/2017. Means followed by different letters (capital letters for main effect of harvest date H and cultivar C; lowercase letters for interaction effect H × C) are significantly different at  $p \leq 0.05$  according to Tukey’s HSD test.



**Fig. 5** Peroxidase activity in two kale cultivars (W – ‘Winterbor’ F<sub>1</sub>; R – ‘Redbor’ F<sub>1</sub>) as affected by harvest date (I – before frost; II – after medium frost; III – after heavy frost) in the seasons 2015/2016 and 2016/2017. Means followed by different letters (capital letters for main effect of harvest date H and cultivar C; lowercase letters for interaction effect H × C) are significantly different at  $p \leq 0.05$  according to Tukey’s HSD test.

significant differences in activity of this antioxidant enzyme were observed for harvest date. An interesting fact was that red-leaved ‘Redbor’ F<sub>1</sub> showed significantly higher peroxidase activity in both cultivation seasons in comparison to the green-leaved cultivar. A particularly large increase in peroxidase activity occurred in the 2015/2016 season after heavy frost, as compared to the initial value (Harvest I), and the difference was 212%.

## Discussion

Most of the analyzed constituents were significantly affected by harvest date due to cold stress, and also differed between the two studied kale cultivars: 'Winterbor' F<sub>1</sub> and 'Redbor' F<sub>1</sub>.

Dry weight content depends on weather conditions and crop cultivar [37]. Frost-resistant plants have tolerance to negative temperatures, which is related to a phenomenon called water crystallization in the intercellular spaces, usually leading to cell dehydration and reduction of tissue water content [38], which was observed in our study. Łata and Wińska-Krysiak [39] determined that 'Redbor' F<sub>1</sub> kale had lower dry weight content (16.3% FW) than 'Winterbor' F<sub>1</sub> (17.7% FW). According to Sikora and Bodziarczyk [40], the green kale contained 16.75–17.39%, on average. In our study, the dry weight content was in the range from 14.01% to 28.76% FW. The differences in the content of this constituent between literature data and our observation can be related to cultivars, harvest dates, and climatic factors.

Sugars in the plant arise as a result of the photosynthesis process, and they are the most important source of energy [21]. An important form of spare sugars in plants is starch, which can be used for nightly energy expenditure or can be stored in amyloplasts [41]. Cierieszko [42] claimed that low temperature may result in an increase in the content of soluble sugars up to tenfold. Sugars can play a vital role as osmoregulators as well as cryoprotectants, that is, they are compounds that protect plants against frost. Our data concerning the increase in soluble sugars in kale plants subjected to medium and heavy frost support the notion that these compounds accumulate under stress conditions and contribute to regulating cell osmotic pressure.

Low temperatures belong to the group of stress factors that increase the production of reactive oxygen species [14]. Plants defend against ROS by producing compounds that are antioxidant scavengers, which include, among others, L-ascorbic acid [43]. An increase in the content of L-ascorbic acid after medium frost showed involvement of this compound in antioxidant defence. The decrease in L-ascorbic acid after very strong frost may be related to depletion of the plant's defense system and plant freezing injury. As we observed, a significantly greater decrease in the content of this component at Harvest III in comparison to Harvest II was found in 'Redbor' F<sub>1</sub> than in 'Winterbor' F<sub>1</sub> in the 2016/2017 season when severe frost occurred before Harvest III. Such a decrease was probably caused by frost damages of red-leaved kale, observed visually on the plants of that cultivar, proving its lower resistance to very low temperatures in comparison to 'Winterbor' F<sub>1</sub>. Sikora and Bodziarczyk [40] showed that 'Winterbor' F<sub>1</sub> kale may contain L-ascorbic acid in the range 52.25 to 77.91 mg 100 g<sup>-1</sup> FW, depending on the cultivation year. We determined much higher L-ascorbic acid contents in kale than the above-mentioned authors. Korus [44] reported that there were on average 102 mg of vitamin C in 100 g FW in kale, and this value is more similar to our data. The content of L-ascorbic acid in our experiment was higher for 'Redbor' F<sub>1</sub> in comparison to 'Winterbor' F<sub>1</sub> but only in the season 2015/2016. However, data published by Łata and Wińska-Krysiak [39] showed that the green-leaved kale cultivar was characterized by a higher content of ascorbate than the red-leaved one.

Low temperature usually inhibits chlorophyll accumulation or leads to degradation of these pigments [10,45], which was confirmed in our study. We found a significant decrease in chlorophyll *a* level after medium and heavy frost in the 2015/2016 season and in chlorophyll *a* and *b* concentrations due to heavy frost in the next season. Kale, together with spinach and parsley, belongs to a group of vegetables that are particularly rich in chlorophylls. Korus and Lisiewska [46] showed that the total chlorophyll content in kale ranged from 81 up to 165 mg 100 g<sup>-1</sup> FW. According to the data published by Śniegowska and Biesiada [47], kale contained the most chlorophyll *a+b* (182 mg 100 g<sup>-1</sup> FW) among other brassicas. Korus and Kmiecik [48] showed that the content of chlorophylls in kale was 118–145 mg 100 g<sup>-1</sup> FW. *Brassica* crops, and especially kale, are also rich in carotenoids. Biegańska-Marecik and Radziejewska-Kubzdela [6] confirmed that kale contained larger amounts of carotenoids than other *Brassica* crops or leafy vegetable crops such as broccoli, Brussels sprouts, green lettuce, and spinach; however, it had a similar value of carotenoids to fennel or parsley. Śniegowska and Biesiada [47] observed that the content of carotenoids in kale was 39.55 mg 100 g<sup>-1</sup> FW, on average. Ligor [45] indicated a relationship between the temperature during the cultivation

period and the average content of carotenoids in the plants. The above-mentioned author found a higher content of carotenoids at 15–30°C than at lower temperature (10–20°C). However, our results showed more carotenoids in kale leaves after frost, but only in the season 2015/2016. When frost was more severe (2016/2017 season, Harvest III), carotenoids declined and their content was significantly lower than in kale leaves harvested earlier.

Phenolics are one of the most important antioxidants in plants, and thus an increase due to an abiotic stressor should be expected [14]. Changes in the level of phenolic compounds can occur due to extreme temperatures, drought, high light, etc. We observed that medium and heavy frost significantly increased the content of phenolics (for both cultivars) and anthocyanins (for 'Redbor' F<sub>1</sub>) in both cultivation seasons. This is a confirmation that phenolic compounds play an important role in plant defense mechanism against cold stress. The research conducted by Łata and Wińska-Krysiak [39] confirmed our results that 'Redbor' F<sub>1</sub> had a higher content of phenolic compounds than 'Winterbor' F<sub>1</sub>. According to Sikora and Bodziarczyk [40], kale is characterized by the highest content of phenolics (94.40 mg 100 g<sup>-1</sup> FW) among *Brassica* vegetables, while Brussels sprouts ranked second. The studies of Śniegowska and Biesiada [47] showed that kale had very high antioxidant activity which can be due to phenolic and anthocyanin contents. This was confirmed in the present study, in which the red-leaved 'Redbor' F<sub>1</sub> had 64.71–77.19% of DPPH• scavenging ability, while for 'Winterbor' F<sub>1</sub> it was lower (61.96–62.69%).

Pawłowska and Treder [27] and Wojtyła et al. [43] showed that the activity of peroxidase increased rapidly due to plant response to abiotic stress, including also cold stress. Although in the 2015/2016 season we observed an increase in peroxidase activity after heavy frost, it was not confirmed in the next season. Moreover, peroxidase activity was lower (2015/2016) or similar (2016/2017) to that of the initial values (Harvest I) after moderate frost, which is rather difficult to explain.

## Conclusions

Subzero temperature during the last two harvests had a significant influence on the biological value and physiological status of the tested kale cultivars 'Winterbor' F<sub>1</sub> and 'Redbor' F<sub>1</sub>. It was shown that medium and heavy frost affected dry weight, soluble sugars, phenolics, photosynthetic pigments content, as well as total antioxidant and peroxidase activity. After medium frost, a significant increase was observed in dry weight, soluble sugars content, and antioxidant activity (for both cultivars). Heavy frost caused for both cultivars an increase in the content of dry weight, soluble sugars, phenolics, as well as promoted DPPH• scavenging and peroxidase activity (peroxidase activity increased only in the 2015/2016 season). The anthocyanins content in 'Redbor' F<sub>1</sub> also increased as an effect of cold. Harvest date together with accompanying negative temperatures may regulate L-ascorbic content, and the highest increase in its content was observed after medium frost. The 'Redbor' F<sub>1</sub> cultivar had a much higher content of all biologically active compounds, except for sugars, in comparison to 'Winterbor' F<sub>1</sub>. Frost induced cold acclimatization that affect the nutritional and health-promoting quality of kale. This means that it may be more favorable to harvest kale leaves after exposure to low negative temperatures. We hope that our data also extend knowledge about the physiological background of the response of crops to cold stress.

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### **Stres wywołany mrozem modyfikuje związki bioaktywne w odmianach jarmużu podczas jesienno-zimowych zbiorów**

#### **Streszczenie**

Jarmuż to znana i ceniona od czasów starożytnych roślina warzywna o wysokich walorach prozdrowotnych, wykorzystywana do celów kulinarnych, dekoracyjnych, ale także leczniczych. Celem prezentowanych badań była analiza stanu fizjologicznego i składu odżywczego dwóch odmian jarmużu: 'Winterbor' F<sub>1</sub> (niebiesko-zielone liście) i 'Redbor' F<sub>1</sub> (czerwono-purpurowe liście), które zbierano w trzech terminach: przed wystąpieniem mrozu (>0°C), po umiarkowanym mrozie (-5.0°C) oraz po bardzo ostrym mrozie (-15.0°C). W liściach jarmużu oznaczono zawartość suchej masy, cukrów rozpuszczalnych, kwasu L-askorbinowego, karotenoidów, chlorofilu, polifenoli i antocyjanów, a także aktywność przeciwutleniającą i aktywność peroksydazy. Niska temperatura znacząco wpłynęła na bioaktywne związki występujące w jarmużu. Zawartość suchej masy, cukrów rozpuszczalnych, kwasu L-askorbinowego, fenoli oraz aktywność antyoksydacyjna wzrosła po umiarkowanym mrozie u obu odmian tego warzywa. Poziom antocyjanów także uległ zwiększeniu u odmiany 'Redbor' F<sub>1</sub>. Po bardzo silnym mrozie większość badanych parametrów (zawartość suchej masy, cukrów rozpuszczalnych, związków fenolowych, antocyjanów jak i całkowita aktywność antyoksydacyjna i aktywność peroksydazy) znacznie wzrosła. Zawartość chlorofilu *a* zmniejszyła się w wyniku silnych mrozów w obu sezonach uprawowych. Przeprowadzenie zbioru jarmużu przed lub po wystąpieniu ujemnej temperatury wiąże się z innym poziomem biologicznie czynnych składników, a stan fizjologiczny roślin jest silnie uzależniony od oddziaływania niskiej temperatury.