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Influence of Low-Temperature Stress on the Changes in the Composition of Grapevine Leaf Phenolic Compounds and their Antioxidant Properties

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ABSTRACT

The impact of low-temperature stress on the content of phenolic compounds of grapevine leaves and their antioxidant activity was examined. The experiment with seedlings was performed under optimal conditions and under low-temperature stress. Phenolic compounds were extracted from leaves of *Vitis vinifera* using 80% (v/v) acetone. After lyophilization, the extracts were characterized by their content of total phenolics, condensed tannins, and individual phenolic acids. The reducing power and antiradical activity against DPPH$^{\bullet}$ as well as ABTS$^{\bullet+}$ were also investigated. Low-temperature stress decreased the content of total phenolics and condensed tannins in the leaf extract, but the quantity of some phenolic acids (i.e., gallic, caffeic, and ferulic acids) was higher than in the control extract. Caffeic acid was determined to be the dominant phenolic acid. Although phenolic acids were present in free, esterified, and glycosylated forms, the majority were esterified. The extract of the leaves treated with low-temperature stress was characterized by a lower total antioxidant activity, antiradical activity against DPPH$^{\bullet}$, and reducing power (e.g., the total antioxidant activity of the extract after low-temperature stress was 0.545 mmol Trolox/g extract whereas the extract from unstressed leaves was 0.808 mmol Trolox/g extract).

Keywords: antioxidant activity, grapevine leaves, low temperature, phenolic compounds
Abbreviations: ABTS$^{\bullet+}$, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation; **CAT**, catalase; **DPPH$^{\bullet}$**, 2,2-diphenyl-1-picrylhydrazyl radical; **GR**, glutathione reductase; **HPLC**, high performance liquid chromatography; **ROS**, reactive oxygen species; **RSD**, relative standard deviation; **SOD**, superoxide dismutase; **Trolox**, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; **UV-DAD**, ultra violet - diode array detector

INTRODUCTION

Various environmental and biological factors such as hyperoxia, light, drought, high salinity, cold, metal ions, pollutants, xenobiotics, toxins, reoxygenation after anoxia, experimental manipulations, pathogenic infection and ageing of plants, may induce oxidative stress (Bartosz 1997) and cellular damage, which in turn results in seed deterioration (Bailly 2004). Chilling has an immediate effect on membrane fluidity and enzyme kinetics, thereby influencing many cellular processes (Guy 1990).

Nonetheless, cells are equipped with protecting enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and low-molecular-weight antioxidant compounds that scavenge reactive oxygen species (ROS) and participate in seed survival (Bartosz 1997; Bailly 2004; Farrant 2004). Previous work has shown that low temperature-induced stress can modify the composition of phenolic compounds in different anatomical parts of plants such as pea (Rudikovskaya *et al.* 2008), rapeseed (Solecka *et al.* 1999; Solecka and Kacperska 2003; Stefanowska *et al.* 2002), maize (Christie *et al.* 1994), soybean (Janas *et al.* 2000), winter wheat (Olenichenko *et al.* 2006), elderberry (Thomas *et al.* 2008), *Ammopiptanthus mongolicus* (Liu *et al.* 2007), and *Cotinus coggygria* (Oren-Shamir and Levi-Nissin 1997).

Owing to the fact that scientific information concerning the influence of low-temperature stress on antioxidant capacity of plants is still quite limited, the aim of the present study has been to investigate the changes in phenolic compounds of grapevine leaves and their antioxidant activity, which occur under low-temperature stress and following recovery.

MATERIALS AND METHODS

Plant material

The material for the study consisted of leaves of grapevine *Vitis vinifera*, cultivar *Himrod.* The seedlings from which the leaves were obtained had been purchased from a plant nursery in Jabłonowo (Poland).

Experimental conditions

One-month-old grapevine seedlings (in 2-litre pots) were placed in FITOTRON plant growth chambers made by Snijders Scientific, Tiburg-Holland, model Economic-deluxe 04.1, in which the proper experiment was conducted after two weeks during which the plants were acclimatised under conditions which were optimum for the growth and development of grapevine. During the acclimatisation of plants to the conditions in the growth chambers as well as throughout the whole experiment, soil moisture was maintained at an optimum level, i.e. 70%. To monitor soil moisture, a Moisture Meter made by Mera, model VP-PRL-Nr 279462, was used. The conditions of the experiment are specified in **Table 1**.

One of the growth chambers provided grapevine seedlings with optimum growth conditions (+25°C in the day and +18°C at night), whereas the other was programmed to chill stress conditions (+10°C daytime and +7°C at night). Seedlings kept in the chamber with optimum conditions for the growth of grapevine (+25°C in the day and +18°C at night) were marked as Control 1

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Table 1 Scheme of experiment.

Experimental group	Symbol of group	Experiment duration	Temperature of day	Temperature of night
Control 1	C1	1 week	25°C	18°C
Stress of low temperature	S	1 week	10°C	7°C
Control 2	C2	2 weeks	25°C	18°C
Recovery	S + R	1 week	10°C	7°C
		1 week	25°C	18°C

(C1). Plants which were kept for a week under chill stress conditions (+10°C in the day and +7°C at night) were designated as S. Grapevine seedlings which stayed for two weeks in the growth chamber which had the optimum plant growth conditions (+25°C in the day and +7°C at night) were named Control 2 (C2). Finally, seedlings which remained for a week under chill stress conditions (+10°C in the day and +7°C at night) and for the following week were transferred to optimum growth conditions (+25°C in the day and +18°C at night).

Extraction of phenolic compounds

Phenolic compounds were extracted from the raw material using 80% (v/v) acetone at a solid to solvent ratio of 1:10 (w/v), at 70°C for 15 min (Amarowicz *et al.* 1995). Extraction was carried out in dark-colored flasks using a shaking water bath. The resulting slurries were centrifuged at 4000 × *g* for 15 min, and the supernatant was collected. The residue was re-extracted twice under the same conditions, and supernatants were combined. The solvent was then removed from the combined supernatants under vacuum at 40°C, and the remaining water in the concentrated extract was removed by lyophilization for 72 h at -48°C and 0.046 mbar. Finally, the prepared crude extracts were stored at -20°C in vacuum-sealed pouches (in the dark) until they were used for further analysis.

Determination of total phenolics

The content of phenolic compounds in the extract was determined using the Folin-Ciocalteau's phenol reagent (Naczk and Shahidi 1989) and (+)-catechin was used as a standard.

Determination of condensed tannins

The content of condensed tannins in the extract and leaves was determined using the modified vanillin assay (Price *et al.* 1978) and expressed as absorbance units per 1 g of extract or leaves (A_{500}/g). Briefly, to 0.5 ml of extract 2.5 ml solution B (obtained by dissolving 0.5 g of vanillin in 100 mL of solution A) was added. Solution A was made by supplementing 4 ml of concentrated HCl to 100 ml with methanol. The samples were left in the dark at room temperature for 20 min, and then absorbance was measured at 500 nm.

Separation and analysis of phenolic acids by HPLC

Phenolic acids (i.e. free and those liberated from soluble esters and from soluble glycosides) were isolated from the extract according to the method previously described by Amarowicz and Weidner (2001). An aqueous suspension of the extract (800 mg in 20 ml) was adjusted to pH 2 with 6 M HCl, and free phenolic acids were extracted five times into 20 ml diethyl ether using a separatory funnel. The ether extract was evaporated to dryness under vacuum at room temperature. The water solution was neutralized and then lyophilised. The residue was dissolved in 20 ml 2 M NaOH and hydrolysed for 4 h under a nitrogen atmosphere at room temperature. After acidification to pH 2 using 6 M HCl, phenolic acids released from soluble esters were extracted from the hydrolysate five times into 30 ml diethyl ether. Nine ml 6 M HCl were added to the water solution and the solution was placed in a nitrogen atmosphere and hydrolysed for 1 h in a boiling water bath. Phenolic acids released from soluble glycosides were separated from the hydrolysate five times into 45 ml diethyl ether. After ether evaporation, the dry residue was dissolved in 10 ml methanol and filtered through a 0.45 µm nylon filter. The sample was injected onto an HPLC column. A Shimadzu HPLC system was employed: LC – 10 ADVP pump, photodiode array detector UV-VIS SPD – M10AVP, controller SCL – 10AVP. The conditions of the separations were as follows: pre-packed LUNA C18 column (5 µm, 4.6 × 250 mm; Phenomenex); mobile phase water- acetonitrile - acetic acid (88:10:2, v/v/v) (Amarowicz and Weidner 2001); flow rate 1 ml/min; injection volume 20 µl; the detector was set at 270 and 320 nm. The content of individual phenolic acids was determined using an external standard method. Individual compounds were identified using co-elution with authentic original standards and their UV-DAD spectra.

Analysis of flavonoids

Flavonoids were hydrolysed from glycosides according to Crozier *et al*. (1997). Briefly, 50 mg of the extract was dissolved in 5 ml of 1.2 M HCl in 5% methanol containing 0.2% of TBHQ. Hydrolysis was carried out at 90°C for 2 h. Cooled samples were adjusted to 25 ml with distilled water and analysed by HPLC. The same Shimadzu system was used. The separation was done in a gradient: solvent A water–acetonitrile–acetic acid (93:5:2; v/v/v); solvent B water–acetonitrile–acetic acid (58:40:2; v/v/v); linear gradient from 0 to 100% B for 50 min (Crozier *et al*. 1997); flow rate 1 ml/min; injection volume 20 µl; the detector was set at 360 nm. The content of individual flavonoids was determined using an external standard method. Quercetin was identified using co-elution of authentic standard as well its UV-DAD spectrum.

Determination of total antioxidant activity/capacity (TAA/C)

The total antioxidant activity (TAA) of the extracts was determined according to the Trolox equivalent antioxidant capacity (TEAC) assay as described by Re *et al*. (1999). ABTS cation radical was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand in dark at room temperature for 16 h before use. Prior to assay, the solution was diluted in ethanol to give absorbance at 734 nm of 0.7000 ± 0.020 in a 1 cm cuvette. After the addition of 2.0 ml of diluted $ABTS^{\bullet+}$ to 20 µl of the extract or Trolox standard the sample was incubated for 6 min at 30°C. Then the absorbance at 734 nm was read. The TAA was read from the concentration-response curve for the absorbance at 734 nm for $ABTS^{\bullet+}$ as a function of concentration of standard Trolox solution. The results were expressed as mmol of Trolox equivalents per gram of the extract. Then results were recalculated and TAC was expressed as mmol of Trolox equivalents per gram of leaves.

Determination of DPPH free radical scavenging activity of leaves extracts

The antiradical activity was analyzed with the method described by Yen and Chen (1995). Briefly, 0.008-0.040 mg of an extract dissolved in 0.1 ml of methanol was added to 2 ml of methanol; next, 0.25 ml of a DPPH radical solution in the concentration of 1 mg/ml methanol was added. The mixture was left in the dark at room temperature for 20 min. After that time, absorbance was read at a wavelength of 517 mm. RSD of all measurements were 2-3%.

Determination of reduction power of leaf extracts

Reduction power was determined using the method described by Oyaizu (1986). Briefly, 1 ml water containing 0.2-1.0 mg of an extract was poured to each centrifuge test tube. Then, 2.5 ml of 0.2

Table 2 Content of total phenolics and condensed tannins in extracts and grape leaves.

Compound	C1	S	C2	S+R
Total phenolics				
(mg/g extract)	164.00 ± 2.00 a	111.00 ± 2.00 b	192.00 ± 3.00 c	228.00 ± 3.00 d
(mg/g leaves d.m.)	26.10 ± 0.40 a	19.50 ± 0.30 b	32.00 ± 2.00 c	33.50 ± 0.50 c
(mg/g leaves f.m.)	5.89 ± 0.09 a	4.52 ± 0.07 b	6.73 ± 0.10 c	7.77 ± 0.12 d
Condensed tannins				
(A_{500}/g extract)	44.00 ± 1.00 a	37.00 ± 1.00 b	82.00 ± 2.00 c	113.00 ± 3.00 d
(A_{500}/g leaves d.m.)	7.00 ± 0.16 a	6.48 ± 0.18 b	13.80 ± 0.30 c	16.10 ± 0.44 d
(A_{500}/g leaves f.m.)	1.58 ± 0.12 a	1.48 ± 0.04 a	2.87 ± 0.07 b	3.84 ± 0.10 c

Means ± standard deviations followed by the same letter, within a row, are not significantly different ($p > 0.05$) according to *t*-test.

M phosphate buffer, pH 6.6, and 2.5 ml of 1% solution of potassium ferrocyanide $K_3Fe(CN)_6$ were added. The mixture was incubated in water bath at 50°C for 20 min. Afterwards, 2.5 ml 10% TCA solution was added. From each sample thus prepared, 2.5 ml of the mixture was removed and 2.5 ml of deionized water as well as 0.1% of $FeCl_3$ were added. After 10 min absorbance was measured at 700 nm wavelength. RSD of all measurements were 2-3%.

Statistical analysis

Each extract was considered as a "treatment". Results were expressed as mean ± standard deviation (SD) (n = 3) for each extract. The statistical significance (*t*-test: two-sample equal variance, using two-tailed distribution) was determined using Microsoft Excel statistical software (Microsoft Office Excel 2003, Microsoft Corp., Redmond, WA).

RESULTS AND DISCUSSION

The content of total phenolics in the extract of grape leaves after chill stress (111 mg/g) was significantly lower than in the control extract (164 mg/g) (**Table 2**). Similar findings were observed for results expressed in relation to the dry and fresh matter of leaves. In the extract obtained from the leaf sample after the chill stress and recovery period (S+R) as well as, the leaves themselves, the content of total phenolics was higher than in control (C2) sample. The low-temperature stress also increased the content of condensed tannins in the extract (e.g., the content of tannins in S was 84.1% of that determined in C1), as well in the dry and fresh matter of leaves. The content of total phenolics in grapevine leaves and their extracts determined in this study was high. Durmaz *et al*. (2007) reported lower amounts of total phenolics in some edible leaves. Leafy vegetables such as red lettuce, rough lettuce, red and white cabbage were characterised by the total phenolic content 1.70, 0.53, 0.40, and 1.78 mg/g f.m. (Hassimotto *et al*. 2005). Amarowicz *et al*. (2008b) in leaves from *Vitis vinifera* cv. 'Chasselas rose' determined 41 mg of total phenolic per g of leaves d.m.

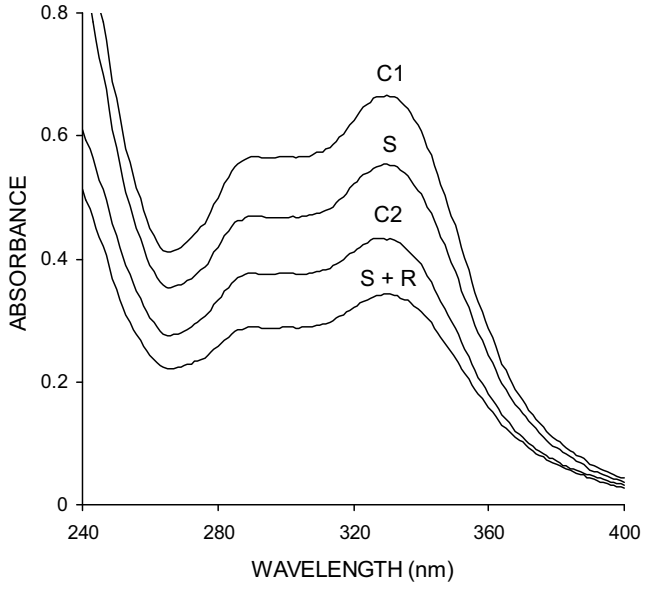

Fig. 1 UV spectra of phenolic compounds present in grapevine leaf crude extracts.

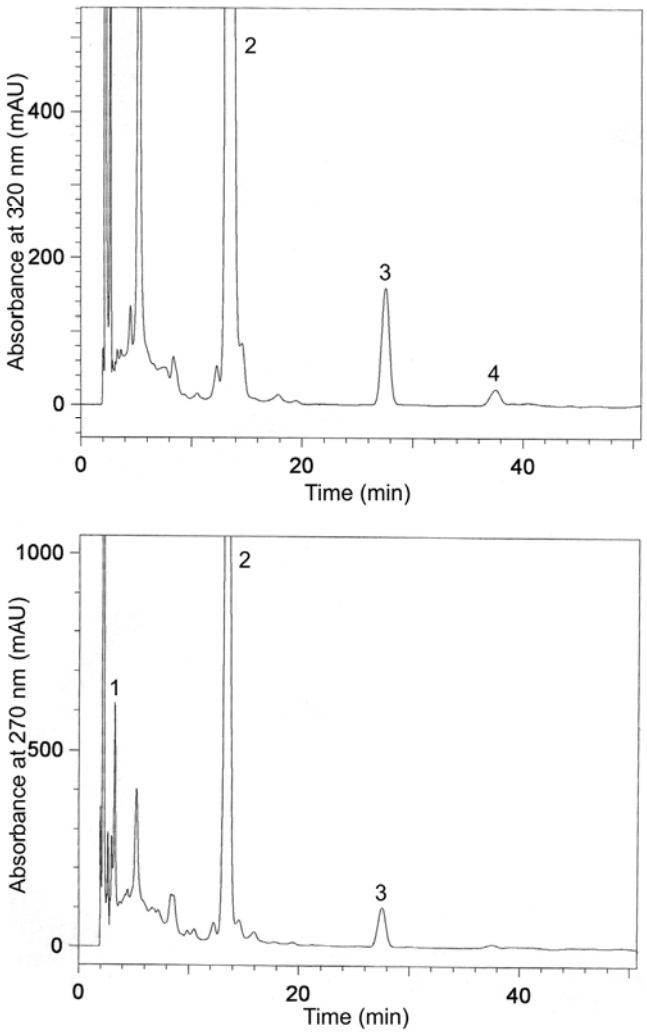

Fig. 2 HPLC chromatogram of phenolic acids from grapevine leaves extract. 1 - gallic acid; 2 - caffeic acid; 3 - *p*-coumaric acid; 4 - ferulic acid.

The UV spectra of phenolic compounds present in all extracts were characterised by maxima at 330 nm and a shoulder at 284 nm (**Fig. 1**). The strong absorption band at 330 nm can be caused by the presence of hydroxycinnamic acid derivatives and flavonols (Mabry *et al*. 1970; Amarowicz and Weidner 2001). The shoulder at the shorter wavelength most probably originated from condensed tannins (Karamać *et al*. 2007; Amarowicz *et al*. 2008a). Similarity in the spectra confirms that chill stress typically only changes the proportions between phenolic compounds present in the grapevine leaves.

The content of gallic, caffeic, *p*-coumaric, and ferulic acids in the extracts and leaves was determined using an HPLC method (**Fig. 2**). Results were also reported in relation to fresh and dry weight of leaves. Caffeic acid was the

Table 3 Content of gallic acid in extracts and grape leaves.

Form of phenolic acid	C1	S	C2	S+R
Free				
(mg/g extract)	-	-	-	-
(mg/g leaves d.m.)	-	-	-	-
(mg/g leaves f.m.)	-	-	-	-
Esterified				
(mg/g extract)	0.201 ± 0.006 a	0.203 ± 0.006 a	0.136 ± 0.004 b	0.367 ± 0.011 c
(mg/g leaves d.m.)	0.032 ± 0.001 a	0.036 ± 0.001 b	0.023 ± 0.001 c	0.054 ± 0.002 d
(mg/g leaves f.m.)	0.007 ± 0.001 a	0.008 ± 0.001 a	0.006 ± 0.001 a	0.016 ± 0.001 d
Glycosylated				
(mg/g extract)	0.058 ± 0.002 a	0.080 ± 0.002 b	0.158 ± 0.005 c	0.097 ± 0.003 d
(mg/g leaves d.m.)	0.010 ± 0.001 a	0.014 ± 0.001 b	0.026 ± 0.001 c	0.014 ± 0.001 b
(mg/g leaves f.m.)	0.002 ± 0.000 a	0.003 ± 0.000 b	0.006 ± 0.000 c	0.003 ± 0.000 b
Total				
(mg/g extract)	0.259 ± 0.008 a	0.283 ± 0.008 b	0.294 ± 0.009 b	0.464 ± 0.014 c
(mg/g leaves d.m.)	0.042 ± 0.001 a	0.050 ± 0.001 b	0.049 ± 0.001 b	0.068 ± 0.002 c
(mg/g leaves f.m.)	0.009 ± 0.001 a	0.011 ± 0.001 b	0.012 ± 0.001 b	0.019 ± 0.001 c

Means ± standard deviations followed by the same letter, within a row, are not significantly different ($p > 0.05$) according to *t*-test.

Table 4 Content of caffeic acid in extracts and grape leaves.

Form of phenolic acid	C1	S	C2	S+R
Free				
(mg/g extract)	0.164 ± 0.005 a	0.127 ± 0.004 b	0.117 ± 0.004 b	0.164 ± 0.005 a
(mg/g d.m.)	0.026 ± 0.001 a	0.023 ± 0.001 b	0.020 ± 0.001 c	0.021 ± 0.001 b
(mg/g f.m.)	0.010 ± 0.001 a	0.005 ± 0.001 b	0.005 ± 0.001 b	0.004 ± 0.001 b
Esterified				
(mg/g extract)	10.685 ± 0.321 a	12.071 ± 0.362 b	8.910 ± 0.267 c	15.497 ± 0.465 d
(mg/g leaves d.m.)	1.693 ± 0.050 a	2.115 ± 0.060 b	1.492 ± 0.040 c	2.275 ± 0.068 b
(mg/g leaves f.m.)	0.382 ± 0.010 a	0.491 ± 0.010 b	0.360 ± 0.010 a	0.553 ± 0.010 c
Glycosylated				
(mg/g extract)	0.181 ± 0.005 a	0.267 ± 0.008 b	0.560 ± 0.017 c	0.416 ± 0.012 d
(mg/g leaves d.m.)	0.029 ± 0.001 a	0.050 ± 0.002 b	0.093 ± 0.003 c	0.060 ± 0.002 d
(mg/g leaves f.m.)	0.010 ± 0.001 a	0.010 ± 0.001 a	0.022 ± 0.001 b	0.010 ± 0.001 a
Total				
(mg/g extract)	11.030 ± 0.331 a	12.465 ± 0.374 b	9.587 ± 0.288 c	15.929 ± 0.478 c
(mg/g leaves d.m.)	1.748 ± 0.052 a	2.188 ± 0.060 b	1.605 ± 0.048 c	2.351 ± 0.070 d
(mg/g leaves f.m.)	0.402 ± 0.010 a	0.506 ± 0.010 b	0.387 ± 0.010 c	0.567 ± 0.010 d

Means ± standard deviations followed by the same letter, within a row, are not significantly different ($p > 0.05$) according to *t*-test.

Table 5 Content of *p*-coumaric acid in extracts and grape leaves.

Form of phenolic acid	C1	S	C2	S+R
Free				
(mg/g extract)	-	-	-	-
(mg/g leaves d.m.)	-	-	-	-
(mg/g f.m.)	-	-	-	-
Esterified				
(mg/g extract)	0.226 ± 0.007 a	0.222 ± 0.007 a	0.206 ± 0.006 b	0.273 ± 0.008 c
(mg/g leaves d.m.)	0.036 ± 0.001 a	0.039 ± 0.001 b	0.034 ± 0.001 a	0.040 ± 0.001 b
(mg/g leaves f.m.)	0.008 ± 0.001 a	0.009 ± 0.001 a	0.008 ± 0.001 a	0.010 ± 0.001 a
Glycosylated				
(mg/g extract)	0.025 ± 0.001 a	0.013 ± 0.002 b	0.043 ± 0.005 c	0.040 ± 0.001 d
(mg/g leaves d.m.)	0.004 ± 0.000 a	0.002 ± 0.000 b	0.007 ± 0.000 c	0.006 ± 0.001 c
(mg/g leaves f.m.)	0.001 ± 0.000 a	0.001 ± 0.000 a	0.002 ± 0.000 a	0.001 ± 0.000 a
Total				
(mg/g extract)	0.251 ± 0.008 a	0.235 ± 0.007 b	0.249 ± 0.007 b	0.313 ± 0.009 c
(mg/g leaves d.m.)	0.040 ± 0.001 a	0.041 ± 0.001 a	0.041 ± 0.001 a	0.046 ± 0.001 b
(mg/g leaves f.m.)	0.009 ± 0.001 a	0.009 ± 0.001 a	0.010 ± 0.001 a	0.011 ± 0.001 a

Means ± standard deviations followed by the same letter, within a row, are not significantly different ($p > 0.05$) according to *t*-test.

dominant phenolic acid found in grapevine leaves and extracts prepared therefrom (**Table 4**). This acid was present in material examined as free, esterified, and glycosylated compounds. Gallic and *p*-coumaric acids were noted only as esters and glycosides (**Tables 3** and **5**). The low-temperature stress resulted in an increase of glycosided gallic acid (**Table 3**) as well as esterified and glycosylated caffeic acid in the extract (**Table 4**). The contents of free caffeic (**Table 4**) and glycosided *p*-coumaric acids in the extract from the leaves after chill stress were higher than in the control group. Data reported in **Table 6** points out the fact that low-temperature stress can generate the synthesis of small amounts of ferulic acid in grapevine leaves in the form of esters and glycosides. The presence of ferulic acid in these same forms was also observed in the leaves after recovery (S + R).

The presence of three flavonoids (**Table 7**) in the extracts of grapevine leaves was confirmed by an HPLC method (**Fig. 3**). Using a standard and UV-DAD spectra data (**Fig. 4**), compound 3 with a retention time of 43.7 min and showing maximum absorbance at 369 nm was identified to be quercetin. UV-DAD spectra of compounds 1 and 2 with retention times of 31.1 and 33.7 min were characterized with maxima at 352 and 354 nm, respectively. These results suggest that compounds 2 and 3 are likely flavonols. In this study the contents of quercetin and two other fla-

Table 6 Content of ferulic acid in extracts and grape leaves.

Form of phenolic acid	C1	S	C2	S+R
Free				
(mg/g extract)	-	-	-	-
(mg/g leaves d.m.)	-	-	-	-
(mg/g leaves f.m.)	-	-	-	-
Esterified				
(mg/g extract)	-	0.085 ± 0.003 a	-	0.075 ± 0.002 b
(mg/g leaves d.m.)	-	0.015 ± 0.001 a	-	0.011 ± 0.001 b
(mg/g leaves f.m.)	-	0.003 ± 0.000 a	-	0.002 ± 0.000 b
Glycosylated				
(mg/g extract)	-	0.025 ± 0.001 a	-	0.042 ± 0.001 b
(mg/g leaves d.m.)	-	0.004 ± 0.000 a	-	0.006 ± 0.000 b
(mg/g leaves f.m.)	-	0.001 ± 0.000 a	-	0.001 ± 0.000 a
Total				
(mg/g extract)	-	0.110 ± 0.003 a	-	0.117 ± 0.004 a
(mg/g leaves d.m.)	-	0.019 ± 0.001 a	-	0.017 ± 0.001 a
(mg/g leaves f.m.)	-	0.004 ± 0.000 a	-	0.003 ± 0.000 a

Means ± standard deviations followed by the same letter, within a row, are not significantly different ($p > 0.05$) according to *t*-test.

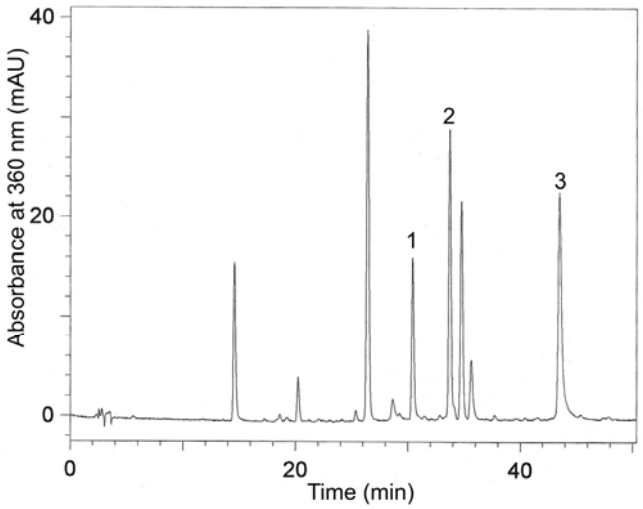

Fig. 3 HPLC chromatograms of flavonoids from grapevine leaves extract. 1 - unknown flavonoid; 2 - unknown flavonoid; 3 - quercetin.

vonoids in the extract and leaves treated with low-temperature stress were higher than in the control group. Kolb and Pfundel (2005) reported the presence of two such derivatives of quercetin and kaempferol in an extract of *Vitis vinifera* grapevine leaves. Myricitin, quercetin, and kaempferol were detected in hydrolysed extracts of *Vitis rotundifolia* grapevine leaves (Pastrana-Bonilla *et al*. 2003).

The extract of leaves treated with low-temperature stress was characterized by a reduced total antioxidant activity: 0.545 mmol Trolox/g extract for S and 0.808 mmol Trolox/g extract for C1 (**Table 8**). The same relationship was observed for the total antioxidant capacity of grape leaves. After recovery (S+R), total antioxidant activity of the extract and the leaves was higher than that from C2. Taking under consideration the changes of total phenolics, condensed tannins, and individual phenolic acids and flavonoids it seems that the decrease of total antioxidant activity in the extracts and leaves was caused in general by a decrease of the content of condensed tannins. A strong antioxidant activity of tannins was confirmed by several authors (Muir 1996; Amarowicz *et al*. 2000; Naczk *et al*. 2003).

All extracts showed antiradical activity against the DPPH radical (**Fig. 5**). The weakest antiradical activity was observed for the extract of leaves with low-temperature stress. The extract of phenolic compounds obtained from the leaves regenerated after stress demonstrated the strongest free radical scavenging activity. The same extract also exhibited the strongest reducing power (**Fig. 6**). The reducing power of phenolic compounds present in the extract

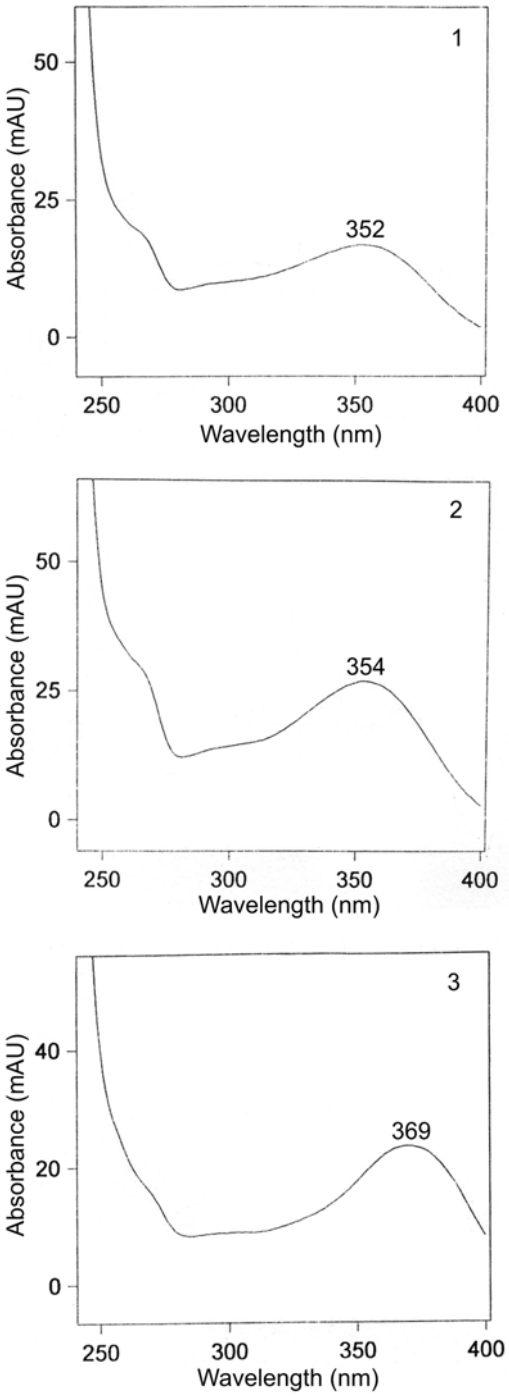

Fig. 4 UV-DAD spectra of flavonoids from grapevine leaves extract. 1-3 - numbers of peaks corresponding to **Fig. 3**.

from the leaves after chill stress (S) was markedly lower than those originating from phenolic compounds of the control (C1) extract.

The changes in the content of total phenolics and individual phenolic constituents of extracts are in accordance with literature data. The cold acclimation of plants resulted in a pronounced increase in phenylalanine ammonia-lyase (PAL) activity (Solecka and Kacperska 1995; Janas *et al*. 2000). In our other experiment (Weidner *et al*. 2009), a significantly reduced content of total phenolic compounds was found in the roots of grapevine seedlings subjected to chill stress. However, the total content of these compounds increased significantly in the roots of plants which underwent recovery after chill stress. A significant increase in the level of condensed tannins in root extracts was found during the recovery process after stress. The weakest capacity to scavenge $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ free radicals and weakest the reducing were shown by the extract obtained from grapevine

Table 7 Content of flavonoids in extracts and grape leaves.

Compound	C1	S	C2	S+R
Compound (1)				
(mg/g extract)	2.810 ± 0.084 a	4.633 ± 0.139 b	2.525 ± 0.016 a	4.845 ± 0.145 b
(mg/g leaves d.m.)	0.444 ± 0.013 a	0.562 ± 0.017 b	0.088 ± 0.003 c	0.712 ± 0.021 a
(mg/g leaves f.m.)	0.101 ± 0.003 a	0.132 ± 0.004 b	0.021 ± 0.001 c	0.174 ± 0.005 d
Compound (2)				
(mg/g extract)	5.151 ± 0.180 a	5.275 ± 0.185 b	5.663 ± 0.198 b	4.173 ± 0.146 c
(mg/g leaves d.m.)	0.814 ± 0.028 a	0.923 ± 0.032 b	0.946 ±0.033 b	0.614 ± 0.021 c
(mg/g leaves f.m.)	0.185 ± 0.006 a	0.216 ± 0.008 b	0.227 ± 0.008 b	0.150 ± 0.005 c
Quercetin				
(mg/g extract)	2.656 ± 0.106 a	3.188 ± 0.128 b	1.508 ± 0.060 c	4.403 ± 0.176 d
(mg/g leaves d.m.)	0.420 ± 0.017 a	0.558 ± 0.022 b	0.252 ± 0.010 c	0.647 ± 0.026 d
(mg/g leaves f.m.)	0.096 ± 0.004 a	0.131 ± 0.005 b	0.060 ± 0.002 c	0.159 ± 0.006 d

Means ± standard deviations followed by the same letter, within a row, are not significantly different ($p > 0.05$) according to *t*-test

Table 8 Total antioxidant activity/capacity of extracts and grape leaves.

Antioxidant activity	C1	S	C2	S+R
(mmol Trolox/g extract)	0.808 ± 0.010 a	0.545 ± 0.010 b	0.858 ± 0.010 c	1.008 ± 0.020 d
(mmol Trolox /g d.m.)	0.129 ± 0.002 a	0.095 ± 0.010 b	0.144 ± 0.002 c	0.148 ± 0.003 c
(mmol Trolox /g f.m.)	0.029 ± 0.001 a	0.022 ± 0.001 b	0.030 ± 0.001 a	0.034 ± 0.001 c

Means ± standard deviations followed by the same letter, within a row, are not significantly different ($p > 0.05$) according to *t*-test.

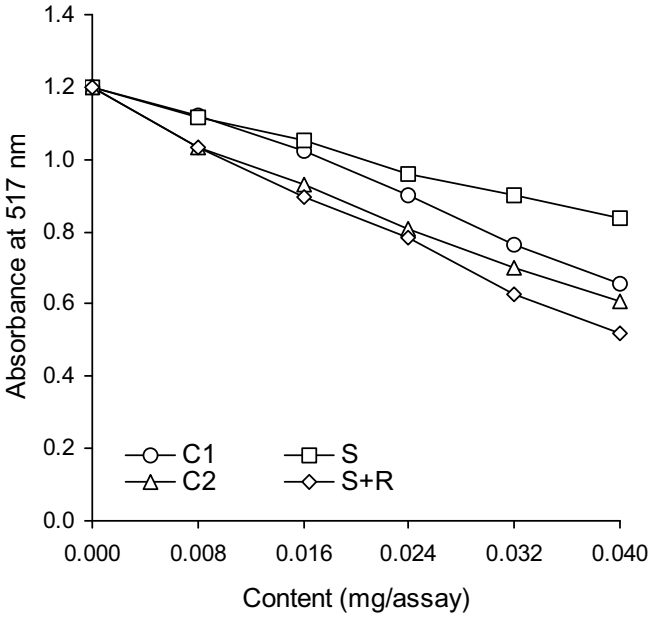

Fig. 5 Antiradical activity of grape leaves extracts against DPPH radical.

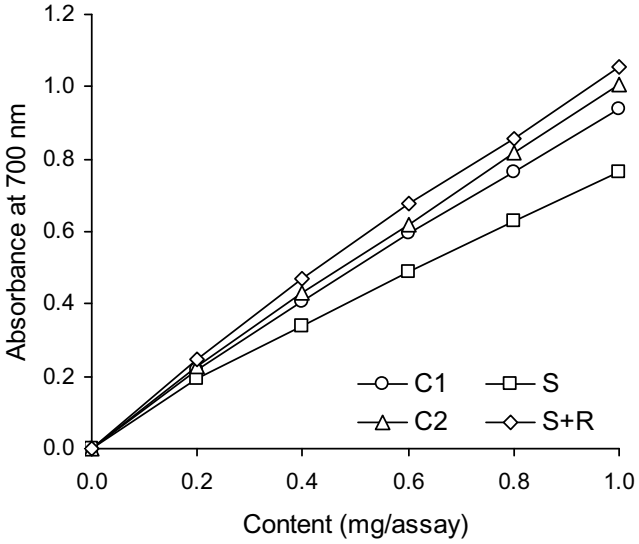

Fig. 6 Reducing power of grape leaves extracts.

roots of the seedlings subjected to chill stress. In the experiments of Rudikovskaya *et al*. (2008), it was shown that, in pea (*Pisum sativum* L.) grown at low temperature (8°C), that the flavan content in roots during the early growth phase was lower than in plants grown at 22°C; later the content increased and was 40% greater than that in plants grown at 22°C (flavan was a dominating component of the soluble phenol fraction). The results of Liu *et al*. (2007) showed that the freezing tolerance of *Ammopiptanthus mongolicus* (*i.e*., evergreen broad-leaved shrub, local species of the Alashan desert, northwest sand area of China) seedlings increased after acclimation at 2-6°C for 14 days, and was accompanied by an increase in the content of phenolic compounds in the cotyledons. Cytochemical observation showed that phenolic deposits were mainly localized in vacuoles and in close proximity to tonoplasts, and also in the cytoplasm. Low temperatures induced accumulation of anthocyanins in *Cotinus coggygria* leaves. Both young and fully matured leaves of plants grown in 17°C/9°C day/night cycles accumulated high concentrations of anthocyanins, compared with plants grown at higher temperatures (Oren-Shamir and Levi-Nissin 1997). Acclimation of winter rapeseed in the cold (i.e., at temperatures >0°C) followed by short exposure to sublethal freezing temperatures resulted in large phenolic deposits in leaves in the vicinity of the plasma membrane and membrane-bound organelles, such as chloroplasts, large vesicles or cytoplasm/tonoplast interfaces (Stefanowska *et al*. 2002). Acclimation of rapeseed in cold and the frost-thaw treatment resulted in the promotion of phenolic esterification. High rates of accumulation of ferulic and sinapic acids in cold were associated with their almost complete esterification under these temperature conditions, whereas freezing treatment was required for increased esterification of *p*-coumaric and caffeic acids, the accumulation of which was lower in leaves of plants grown for 3 weeks at 2°C (Solecka *et al*. 1999). Analyses of different fractions of phenolic acids separated from soybean roots showed that during 24 h of low temperature exposure, an increase in the relative level of ester-bound-soluble phenolic acids occurred. The highest increase in this fraction was observed for ferulic acid (26%). At the same time, a decrease in phenolic glycosides took place (Janas *et al*. 2000). Low temperature induced the accumulation of phenolic compounds (phenolic acids and flavonoids) in winter wheat (*Triticum aestivum* L.) leaves, whereas their qualitative composition was not practically changed (Olenichenko *et al.* 2006).

CONCLUSIONS

The results of this study have demonstrated that the content of total phenolics and condensed tannins significantly fell

as a result of low temperatures but increased during recovery after chill stress. The weakest ability to scavenge $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ free radicals as well as the reducing power were shown by the extract obtained from grapevine leaves from the seedlings subjected to chill stress. Both free radical scavenging activity and reducing power were observed to increase considerably during recovery after stress. This seems to prove that during the recovery process following chill stress the synthesis of antioxidative compounds in grapevine leaves is much more intensive.

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REFERENCES

Amarowicz R, Piskuła M, Honke J, Rudnicka B, Troszyńska A, Kozłowska H (1995) Extraction of phenolic compounds from lentil (*Lens culinaris*) with various solvents. *Polish Journal of Food and Nutrition Sciences* **45**, 53-62

Amarowicz R, Naczk M, Zadernowski R, Shahidi F (2000) Antioxidant activity of condensed tannins of beach pea, canola hulls, evening primrose, and faba beans. *Journal of Food Lipids* **7**, 199-211

Amarowicz R, Weidner S (2001) Content of phenolic acids in rye caryopses determined using DAD-HPLC method. *Czech Journal of Food Science* **19**, 201-205

Amarowicz R, Estrella I, Hernández T, Troszyńska A (2008a) Antioxidant activity of extracts of adzuki bean and its fractions. *Journal of Food Lipids* **15**, 119-136

Amarowicz R, Narolewska O, Karamać M, Kosińska A, Weidner S (2008b) Grapevine leaves as a source of natural antioxidants. *Polish Journal of Food and Nutrition Sciences* **58**, 73-78

Bartosz G (1997) Oxidative stress in plants. *Acta Physiologia Plantarum* **19**, 47-64

Bailly C (2004) Active oxygen species and antioxidants in seed biology. *Seed Science and Biology* **14**, 93-107

Christie RJ, Alfenito MR, Walbot V (1994) Impact of low temperature stress on general phenylopropanoid and anthocyanin pathways: enhancement of transcript abundance and anthocyanin pigmentation in maize seedlings. *Planta* **194**, 541-549

Crozier A, Jensen E, Lean MEJ, McDonald MS (1997) Quantitative analysis of flavonoids by reversed-phase high-performance liquid chromatography. *Journal of Chromatography A* **761**, 315-321

Durmaz G, Cam M, Kutlu T (2007) Antioxidany activities of some edible leaves. In: *2nd International Congress on Food and Nutrition*, 24-26 October 2007, Istanbul, Turkey, p 34 (Abstract)

Farrant JM, Bailly C, Leymarie J, Hamman B, Come D, Corbineau F (2004) Wheat seedlings as a model to understand desiccation tolerance and sensitivity. *Physiologia Plantarum* **120**, 1-12

Guy CL (1990) Cold acclimation and freezing stress tolerance: role of protein metabolism. *Annual Review in Plant Physiology* **31**, 187-223

Hassimotto NMA, Genovese MI, Lajolo EM (2005) Antioxidant activity of dietary fruits, vegetables, and commercial frozen fruit pulps. *Journal of Agricultural and Food Chemistry* **53**, 2928-2935

Janas KM, Cvikrova M, Pałągiewicz A, Eder J (2000) Alterations in phenylpropanoid content in soybean roots during low temperature acclimations. *Plant Physiology and Biochemistry* **38**, 587-593

Karamać M, Kosińska A, Rybarczyk A, Amarowicz R (2007) Extraction and chromatographic separation of tannin fractions from tannin-rich plant material. *Polish Journal of Food and Nutrition Sciences* **57**, 471-474

Kolb CA, Pfundel EE (2005) Origin of non-linear and dissimilar relationships between epidermal UV absorbance and UV absorbance of extracted phenolics in leaves of grapevine and barley. *Plant, Cell and Environment* **25**, 580-590

Liu M-Q, Chen Y-Y, Lu C-F, Zhang H, Yin W-L (2007) Cold acclimation induced accumulation of phenolic compounds and freezing tolerance in *Ammopiptanthus mongolicus*. *Forestry Studies in China* **9**, 203-207

Mabry TJ, Markham KR, Thomas MB (1970) *The Systematic Identification of Flavonoids*, Springer-Verlag, New York, pp 41-164

Muir AD (1996) Antioxidative activity of condensed tannins. In: Shahidi F (Ed) *Natural Antioxidants. Chemistry, Health Effects, and Applications*, AOCS Press, Champaign, Illinois, pp 204-212

Naczk M, Shahidi F (1989) The effect of methanol-ammonia-water treatment on the content of phenolic acids of canola. *Food Chemistry* **31**, 159-164

Naczk M, Amarowicz R, Zadernowski R, Pegg R, Shahidi F (2003) Antioxidant capacity of crude phenolic extracts from wild blueberry leaves. *Polish Journal of Food and Nutrition Sciences* **53 SI 1**, 166-169

Olenichenko NA, Ossipow VI, Zagoskina NV (2006) Effect of cold gardening on the phenolic complex of winter wheat leaves. *Russian Journal of Plant Physiology* **53**, 554-559

Oren-Shamir M, Levi-Nissin A (1997) Temperature effects on the leaf pigmentation of *Cotinus coggygria* "Royal Purple". *Journal of Horticulture Science* **72**, 425-432

Oyaizu M (1986) Studies on products of browning reaction-antioxidative activities of products of browning reaction prepared from glucosamine. *Japanese Journal of Nutrition* **44**, 307-315

Pastrana-Bonilla A, Akoh CC, Sellappan S, Krewer G (2003) Pnenolic content and antioxidant capacity of muscadine grapes. *Journal of Agriculture and Food Chemistry* **51**, 5497-5503

Price ML, Van Scoyoc S, Butler LG (1978) A critical evaluation of the vanillic re-action as an asssay for tannin in sorghum grain. *Journal of Agricultural and Food Chemistry* **26**, 1214-1218

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radicals in Biology and Medicine* **26**, 1231-1237

Rudikovskaya EG, Fedorova GA, Dudareva LV, Rudokovski AV (2008) Effect of growth temperature on the composition of phenols in pea roots *Russian Journal of Plant Physiology* **55**, 712-715

Solecka D, Kacperska A (1995) Phenylalanine ammonialyase activity in leaves of winter oilseed rape plants as affected by acclimation of plant to low temperature. *Plant Physiology and Biochemistry* **33**, 585-591

Solecka D, Boudet AM, Kacperska A (1999) Phenylpropanoid and anthocyanin changes in low-temperature treated winter oilseed rape leaves. *Plant Physiology and Biochemistry* **37**, 491-496

Solecka D, Kacperska A (2003) Phenylpropanoid deficiency affects the course of plant acclimation to cold. *Physiologia Plantarum* **119**, 253-262

Stefanowska M, Kuraś M, Kacperska A (2002) Low temperature-induced modifications in cell ultrastructure and localization of phenolics in winter oilseed rape (*Brassica napus* L. var. *oleifera* L.) leaves. *Annals of Botany* **90**, 637-645

Thomas AL, Chen Y-C, Rottinghaus GE, Malone AM, Byers PL, Applequist WL, Finn CE (2008) Occurrence of rutin and chlorogenic acid in elderberry leaf, flower, and stem in response to genotype, environment, and season. *Acta Horticulturae* **765**, 197-206

Yen GC, Chen H-Y (1995) Antioxidant activity of various tea extracts in relation to their antimutagenicity. *Journal of Agricultural and Food Chemistry* **43**, 27-32

Weidner S, Kordala EK, Brossowska-Arendt W, Karamać M, Kosińska A, Amarowicz R (2009) Phenolic compounds and properties of antioxidants in grapevine roots (*Vitis vinifera*) under low-temperature stress followed by recovery. *Acta Societatis Botanicorum Poloniae*, in press