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Physio-biochemical responses of sage genotypes to chilling

Andrzej Kalisz[1], Agnieszka Sękara[1], Robert Pokluda[2], Aleš Jezdinský[2], Jarmila Neugebauerová[2], Aneta Grabowska[1], Rita Jurkow[1], Katalin Angéla Slezák[3]*

[1]*Department of Horticulture, University of Agriculture in Krakow, Kraków, Poland*
[2]*Department of Vegetable Sciences and Floriculture, Mendel University in Brno, Lednice, Czech Republic*
[3]*Department of Vegetable and Mushroom Growing, Szent István University, Budapest, Hungary*

**Corresponding author: andrzej.kalisz@urk.edu.pl*

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Abstract: This study evaluated sage (*Salvia officinalis* L.) genotypes (cultivars: 'Berggarten', 'Icterina', 'Purpurascens', 'Tricolor', local Czech accessions from the Lednice region, South Moravia: 'LDN-1' and 'LDN-2') subjected to chilling (4 °C, 2 weeks, 18 °C – control) for comparison of antioxidant defence systems. Chilling caused the most significant increase in the peroxidase activity in 'Purpurascens' and 'Tricolor', by 108.5% and 15.7%, respectively, while the catalase was unaffected by the low temperature. The phenolics increased in 'Purpurascens' and 'LDN-1' by 17.2% and 18.1%, respectively, and decreased in 'LDN-2' and 'Tricolor', by 10.6% and 11.7%, respectively, as a result of the chilling. In the sage treated with chilling, the scavenging of 2,2-diphenyl-1-picrylhydrazyl radicals (DPPH•) was higher (by 3%, on average), especially in 'Berggarten', 'Icterina', and 'Purpurascens', than in the control. However, the chilled 'LDN-2' and 'Tricolor' showed lower antioxidant activity in comparison to the control. The malondialdehyde remained stable or was higher in the control, with the only exception being 'LDN-1', where its content increased by 11.4% in the chilled sage. In most genotypes, the content of the dry weight increased in the chilled plants by 9.4% on average. The responses of 'Icterina' and 'Purpurascens' to the low temperature was the most significant, but resulted from different physiological mechanisms. 'Purpurascens' showed the highest increase in the peroxidase activity due to the chilling, while the highest increase in the antioxidant activity was observed for 'Icterina'.

Keywords: antioxidants; genotypic variability; *Salvia officinalis*; low temperature

Sage (*Salvia officinalis* L., Lamiaceae) is a plant native to the coastal region of the Mediterranean, but today, due to the economic importance of this plant, it is cultivated in temperate regions all around the world (Lakušić et al. 2013). This species is well known for its antioxidative properties, attributed to phenolic acids, flavonoids, and di- and triterpenoids (Lu, Foo 2002; Szentmihályi et al. 2004; Afonso et al. 2019). It is considered that diterpene the carnosic acid (salvin), rosmarinic acid (phenolic compound and ester of caffeic acid), and their derivatives are mainly responsible for the antioxidant activities of the *Salvia* species (Cuvelier et al. 1996). A strong differentiation in the levels of bioactive compounds

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among the *Salvia* genus or among various genotypes within a particular species has been observed (Szentmihályi et al., 2004; Poulios et al. 2020). Cuceu et al. (2015) described differences between *Salvia officinalis* cultivars in the content of the phenolics and antioxidant activity. They found that the sage 'Tricolor' had around 10% more phenolics than the 'Purpurascens' plants; however, the DPPH• scavenging activity was similar. The bioactive compound composition and concentrations are dependent on a wide range of factors, including the plant genetic background, but also other abiotic and biotic factors. The external temperature is one of the most important stimuli that causes various physiological and metabolic changes in plants (Ruelland, Collin 2012), but the response of the plants to low temperature is dependent on the specific genotype (Kalisz et al. 2016).

Low temperature causes, inter alia, enhanced reactive oxygen species (ROS) production that can lead to cellular damage. To counter this, plants launch defence mechanisms including increasing the antioxidant enzyme activity and synthesising non-enzymatic antioxidants that can protect the cell against damage caused by the ROS (Sharma et al. 2012; Abid, Abid Ali Khan 2019). The enzymatic oxygen-scavenging system comprises superoxide dismutase (SOD), glutathione peroxidase (GPX), guaiacol peroxidase (POX), catalase (CAT) and other enzymes including those of the ascorbate-glutathione (AsA-GSH) cycle (Caverzan et al. 2016; Xu et al. 2010; You, Chan 2015; Hasanuzzaman et al. 2019). CAT, localised largely in the peroxisomes, is responsible for the reduction of H_2O_2 to H_2O and O_2, while peroxidases, localised in the mitochondria, chloroplasts, peroxisomes, and the cytosol, reduce the H_2O_2 and organic hydroperoxides. In contrast to CAT, peroxidases require cellular reducing agents, for example, ascorbic acid, glutathione, thioredoxin, or glutaredoxin (Karuppanapandian et al. 2011; You, Chan 2015). We focused on peroxidases and catalases in sage cultivars, and determining the responses of the enzymatic systems to chilling due to the genetic differences. The effects of abiotic stresses on antioxidant enzyme activity has been confirmed in several reports and reviews (Gill, Tuteja 2010; Guo et al. 2012; Kusvuran et al. 2016, and others), with some evidence of the up-regulation of particular enzymes and the down-regulation of others at the same time point (Lee, Lee 2000; Kusvuran et al. 2016). However, the genotypic variability, in this respect, has been poorly described. The chilling treatment (6 °C for 8 days) resulted in higher peroxidase activity in Thai and green basils, and changes in the CAT activity was negligible for all six genotypes, with the exception of Thai basil for which the CAT activity dropped (Kalisz et al. 2016). Guo et al. (2006) found that activities of antioxidant enzymes (SOD, CAT, and ascorbate peroxidase – APX) were enhanced greatly until 3 days after the chilling stress in two chilling-tolerant rice cultivars and decreased 5 days after chilling. On the other hand, the activities of antioxidant enzymes were decreased in chilling-sensitive cultivars after the chilling stress.

Non-enzymatic compounds form the other part of the antioxidant machinery, comprising lipid-soluble membrane-associated antioxidants (e.g., α-tocopherol and β-carotene) and water-soluble reductants (e.g., glutathione, phenolics and ascorbate) (Caverzan et al. 2016; Kusvuran et al. 2016). The phenolic compounds of sage have revealed an excellent scavenging activity of the ROS, such as superoxide anion radicals, hydroxyl radicals, and singlet oxygen (Sharma et al. 2012; Naikoo et al. 2019). The phenolic levels in plants can be altered through a chilling treatment, as was described by Rivero et al. (2001) and by Oh et al. (2009), but changes in the phenolics under stressful conditions can be also genotype dependent. Lee and Oh (2015) treated kale cultivars with 4 °C for 3 days and they found that 'Manchoo Collard' had a 15% higher total phenolic concentration than the control after 2 days of recovery, whereas that of the 'TBC' cultivar was 16% lower than that of the control. Kalisz et al. (2016) reported that five tested basil cultivars did not show significant increases in the total phenolic contents after chilling (6 °C for 8 days), but lettuce leaf basil contained more than 60% of these constitutes in comparison to the non-chilled control plants of that genotype. The antioxidant capacity can be measured via the DPPH• radical scavenging activity, which reflects the scale of the involvement of non-enzymatic antioxidants in the defence of plants against stress. The DPPH• scavenging activity is positively correlated with the contents of the phenolic compounds in plants (Wojdyło et al. 2007; Oh et al. 2009; Kalisz et al. 2016). Similar to the level of many antioxidant compounds, the DPPH• scavenging activity depends on the genotype of the plant. Kalisz et al. (2016) reported that the DPPH• scavenging activity was higher in some chilled basil

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cultivars (red basil, lettuce leaf basil, and cinnamon basil), while, for other basil cultivars, it remained at a similar level to the control.

We hypothesise that the sage genotypes investigated in the present study can activate defence mechanisms against low temperature stress in different ways. These differences may be reflected by the different times of the activation of the antioxidant enzymes, the different durations of this activation and the greater or lesser roles of the non-enzymatic antioxidants in neutralising the ROS. We also determined the dry weight and malondialdehyde contents as auxiliary parameters for assessing plant reactions to the temperature stress. We expect that the analyses performed in the present experiment will provide important new insights into the responses of sage cultivars to chilling, and will help to select a genotype with high oxidation scavenging ability.

MATERIAL AND METHODS

Plant material and experimental layout. Plants of the *Salvia officinalis* L. (Lamiaceae) cultivars/accessions: 'Berggarten', 'Icterina', 'Tricolor' from the Zielone Progi nursery centre (Poland), 'Purpurascens' and 'LDN-1' from Školky Litomyšl (Czech Republic), and 'LDN-2' from PERENY (Czech Republic) were used in the experiment conducted in 2017. Cultivar characteristics: 'Berggarten' (more compact growth than the others, wide oval leaves are silvery-blue), 'Icterina' – yellow-green and light green variegated leaves, 'Tricolor' – with greyish green leaves marbled with white, pink and purple, 'Purpurascens' – a purple-grey foliage, and two local Czech accessions (Lednice region, South Moravia): 'LDN-1' – leaves are greenish-grey, oblong, 'LDN-2' – leaves variegated with yellow and light green). All the plants were grown in 1 dm^3 pots in a TS1 peat-based substrate (Klasmann-Deilmann, Germany). The first group of plants was subjected to chilling (constant 4 °C for 2 weeks) and the second group was the control (18 °C). The day length was 12 h, a canopy level irradiance intensity of approximately 380 μmol/m^2·s was provided by an LED lamp (Best LED I panel light, Czech Republic), and the relative humidity was ca. 75%. All the fully developed and healthy leaves from thirty plants of each cultivar (plants with a height of 15–20 cm) were sampled in July (10 plants per replicate), and then they were submitted for the laboratory analysis.

Determination of dry weight. The dry weight (DW) was determined by the gravimetric method according to Pijanowski et al. (1964). The plant samples were dried at 65 °C until a constant mass was achieved, after which the weights of the dried and fresh samples were measured using a Sartorius A120S (Sartorius AG, Germany).

Determination of malondialdehyde content. The level of the lipid peroxidation was measured by estimating the malondialdehyde (MDA) content, using thiobarbituric acid (TBA) as the reactive material, following Dhindsa and Matowe (1981). The tissues were homogenised with 0.1% trichloroacetic acid (TCA). A K-phosphate buffer (pH 7.6), and 0.5% thiobarbituric acid in 20% trichloroacetic acid were added to the supernatant. The reaction mixtures were heated to 95 °C until an orange colour was obtained. The absorbance of the obtained solution was measured at λ = 520 nm and λ = 600 nm (UV-VIS Helios Beta spectrophotometer, Thermo Fisher Scientific Inc., USA). The amount of the MDA was calculated from the difference in the absorbance at these wavelengths, using an extinction coefficient of 155 mM/cm. For the reference, 0.1% TCA was used instead of the supernatant.

Antioxidant enzyme assays in sage leaves. To determine the enzyme activity, the plant material (2.5 g of fresh leaves) was ground in an ice bath with 20 cm^3 of a 0.05 M potassium phosphate buffer and centrifuged (3 492 *x* g, 15 min, 4 °C). Then, the peroxidase (POD) activity was assayed, as described by Lück (1962), with *p*-phenylenediamine as the electron donor substrate and hydrogen peroxide (H_2O_2) as the oxidant. The reaction mixture contained the diluted supernatant, 0.05 M potassium phosphate buffer, *p*-phenylenediamine, and hydrogen peroxide (H_2O_2). The changes in the absorbance at 485 nm were recorded at 60-s intervals for 2 min using a UV-VIS Helios Beta spectrophotometer (Thermo Fisher Scientific Inc., USA). The POD activity was expressed in units (U) per gram fresh weight (FW) per minute. One unit is defined as an increase in the absorbance by 0.1 per minute. The catalase (CAT) activity was measured according to the method described Aebi (1984) by spectrophotometrically (UV-VIS Helios Beta spectrophotometer) monitoring the decrease in the absorbance at 240 nm resulting from the decomposition of H_2O_2. Test tubes contained 1.8 cm^3 of the 0.05 M phosphate buffer (pH 7.0) and 1.0 cm^3 of the 0.05% H_2O_2 solution in the 0.05 M potassium phosphate buffer (pH 7.0). Next, 0.2 cm^3 of the supernatant was added

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every minute for 5 min, and the decrease in the absorbance against a blank was measured. The CAT activity was defined as 1 mmol H_2O_2 decomposed per minute per gram FW.

Quantification of total phenolic content. The total phenolics were estimated using the modified Folin-Ciocalteu colorimetric method (Djeridane et al. 2006). The leaf samples were collected from the experimental plants of each treatment, and each sample (2.5 g) was mixed with 10 cm^3 of 80% methanol. Then, the samples were centrifuged for 15 min at 3492 *x* g at 4 °C. The glass tubes contained 0.1 cm^3 of the supernatant and 2 cm^3 of sodium carbonate. After 5 min, 0.1 cm^3 of Folin-Ciocalteu's reagent, mixed with deionised water (1 : 1 v/v) was added to the test tubes. The mixture was allowed to stand for 45 min and the phenols were determined by colorimetry at 750 nm on a UV-VIS Helios Beta spectrophotometer (Thermo Fisher Scientific Inc., USA) against a reference solution. The total phenol value was expressed as gallic acid equivalents (mg GAE) per gram FW.

Evaluation of the DPPH• scavenging activity. The total antioxidant activity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a free radical. The absorbance at λ = 517 nm of the supernatant and 0.1 mM DPPH in 80% methanol was measured after 15 min of incubation in darkness at 20–22 °C with the UV-VIS Helios Beta spectrophotometer (Thermo Fisher Scientific Inc., USA). The test tubes contained 0.1 cm^3 of the supernatant and 4.9 cm^3 of 0.1 mM DPPH dissolved with 80% methanol. The antioxidant activity was calculated by the following formula:

$$DPPH\ (\%) = ((A0 - A1) / A0) \times 100$$

where: DPPH• – the antioxidant activity, A0 – the absorbance of the reference solution; A1 – the absorbance of the test solution (Molyneux 2004).

Statistical Analysis. All the values per cultivar were expressed as the means of three replicates ± SD. The statistical analyses were performed by an analysis of variance (two-way ANOVA) using Statistica 13.0 (Dell Inc., USA). The significance levels are reported as significant at $P \leq 0.05$ (*), $P \leq 0.01$ (**), or $P \leq 0.001$ (***) and non-significant (ns) according to Tukey's HSD (honest significant difference) test. Homogenous groups were created using this test at $P \leq 0.05$. The experimental results were examined for Pearson's correlation coefficient (*r*) of the total phenolics and DPPH• scavenging activity. The principal component and cluster analyses were used to assess the similarities and differences among the low temperature treated sage genotypes. For this, the data were standardised and subjected to the multivariate analysis principal component analysis (PCA) using Statistica software to assess the genetic diversity in response to the chilling. PC1 and PC2, which had eigenvalues of 40.72 and 34.73, respectively, were used for a further analysis. A cluster analysis, based on the results, was carried out using Ward's method and Euclidean distance, and the variables were standardised.

RESULTS AND DISCUSSION

The peroxidase (POD) activity generally increased by 25%, on average, in the leaves of the sage plants in response to the chilling when compared to the control (Figure 1). The low temperature was shown to enhance activity of the different ROS-scavenging enzymes, including the peroxidases (Suzuki, Mittler 2006; Sharma et al. 2012; Rezaie et al 2020). Lee and Lee (2000) described a higher activity of ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) in leaves of chilled cucumbers. Similarly, Guo et al. (2012) observed an increase in the level of the peroxidase enzyme activity in chilled peppers, and Rezaie et al. (2020) noted a higher activity of guaiacol peroxidase in basil plants treated with a low temperature. These reports indicate a crucial role of peroxidases in the management of ROS in plants subjected to stress. In the present study, there were genotype × temperature interactions for the POD activity, which showed a differentiated response in the sage genotypes to chilling. The 'Purpurascens' and 'Tricolor' cultivars exhibited the highest increase in POD activity, by 108 and 16% in comparison to the control plants. Among the sage genotypes, the 'Tricolor' sage had an elevated level of POD, while the lowest POD activity was found in 'Berggarten' (Table 1). The genotypic diversity in the POD response to the chilling treatment was also described by Kalisz et al. (2016) and Xu et al. (2010). As was noted by Kang and Saltveit (2002) and Shen et al. (1999), higher antioxidant enzyme activities, including peroxidases, may correspond with a greater tolerance of particular plant genotypes to abiotic stress. However, we did not find any significant effect of chilling on the CAT activity in the sage (Figure 1). Only the non-chilled sage plants of 'LDN-2' and 'Berggarten' differed sig-

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Table 1. Average peroxidise and catalase activity of the sage genotypes regardless of the chilling temperature

Means for sage genotype	Peroxidase activity (U/g FW)	Catalase activity (μmol H_2O_2/min·g FW)
Berggarten	100.3 ± 26.94^{A}	3.677 ± 0.568^{A}
Icterina	145.7 ± 12.16^{B}	4.045 ± 1.085^{A}
Purpurascens	217.0 ± 83.65^{C}	4.506 ± 1.072^{A}
Tricolor	490.3 ± 49.69^{E}	4.597 ± 0.573^{AB}
LDN-1	297.0 ± 21.38^{D}	4.965 ± 0.857^{AB}
LDN-2	323.7 ± 28.21^{D}	6.254 ± 0.828^{B}
Level of significance	***	**

The means within a column followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's HSD test; the level of significance (*$P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; ns non-significant) is also shown; ± standard deviation (SD)

nificantly in the CAT activity, but the differences, in comparison to the chilled plants of these genotypes, were non-significant. A separate statistical analysis for the genotype (main effect) showed a higher CAT level in 'LDN-2' (70% higher than in 'Berggarten', and 39–55% higher than in 'Purpurascens' and 'Icterina', respectively) (Table 1). CAT is one of the most active catalysts in the removal of H_2O_2 (Gill, Tuteja 2010). In plants subjected to abiotic stress, a higher CAT activity was noted by Kuk et al. (2003), Posmyk et al. (2005) and Kanase et al. (2019). However, the variable response of CAT has been observed between plant genotypes under low temperature stress (Shen et al. 1999; Lee, Lee 2000; Xu et al. 2010; Kalisz et al. 2016). We suspected that the CAT was the most active in the sage in the early phase of the stress response, while the PODs are predominant in the acclimation stage, which is in agreement with the data published by Karpinsky et al. (2002).

In the present study, the dry weight (% FW) was higher after the chilling treatment when compared to the control (Table 2). Such increases were confirmed for almost all the tested sage genotypes, and the highest difference was found for the chilled and non-chilled 'Berggarten' and 'Purpurascens'. In this experiment, the chilling duration and temperature level was enough to trigger some differences in the dry weight content of the plants. At the same time, the results indicated less hydration of the tissues of some chilled genotypes, which is

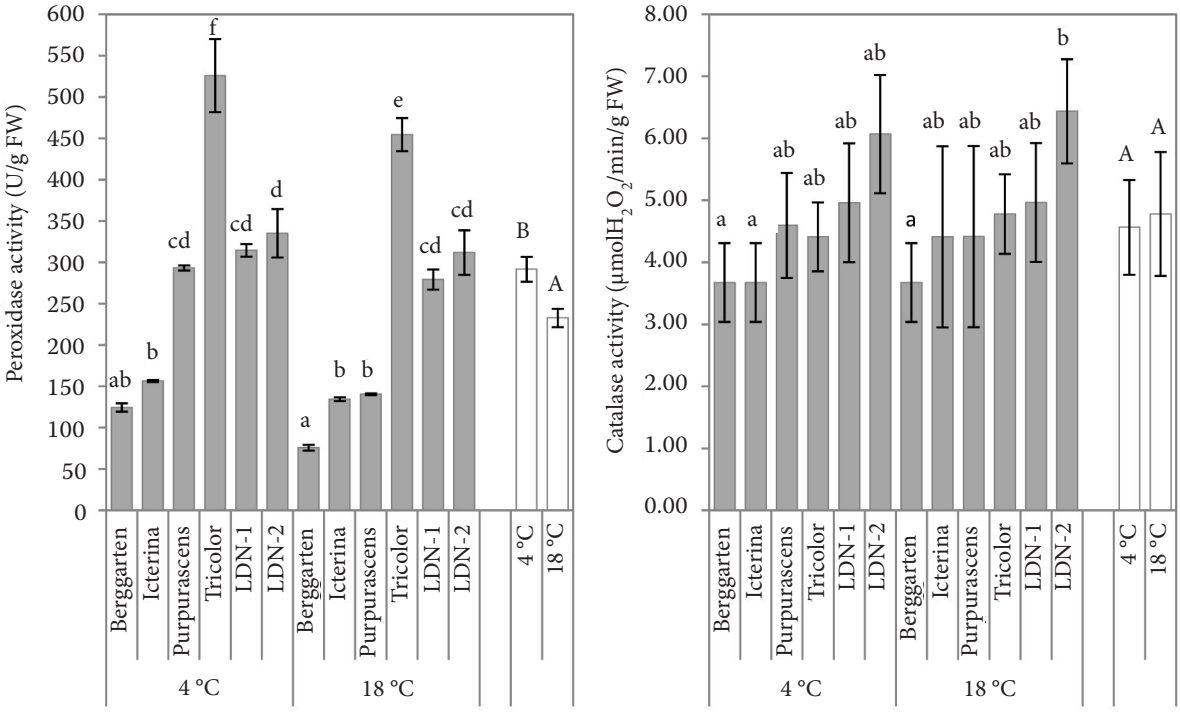

Figure 1. Peroxidase and catalase activity in the sage cultivars as affected by the chilling temperature; the means followed by the different lower case letters for the interaction and the capital letters for the main effect of the temperature are significantly different at $P \leq 0.05$ according to Tukey's HSD test; the bars represent the standard deviations (± SD)

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Table 2. Dry weight and malondialdehyde content in the leaves of sage genotypes subjected to chilling treatment

Temperature	Sage genotype	Dry weight (% FM)	Malondialdehyde (μmol/g FM)
4 °C	Berggarten	17.64 ± 0.193^{b}	7.51 ± 0.526^{a}
	Icterina	17.63 ± 0.193^{b}	9.81 ± 0.455^{b}
	Purpurascens	33.18 ± 0.233^{g}	14.11 ± 1.032^{c}
	Tricolor	17.61 ± 0.045^{b}	10.50 ± 0.298^{b}
	LDN-1	28.43 ± 0.293^{f}	22.88 ± 0.688^{g}
	LDN-2	24.73 ± 0.322^{d}	14.97 ± 0.620^{cd}
18 °C	Berggarten	13.28 ± 0.070^{a}	7.17 ± 0.695^{a}
	Icterina	17.11 ± 0.105^{b}	9.98 ± 0.172^{b}
	Purpurascens	29.11 ± 0.068^{f}	16.57 ± 1.268^{de}
	Tricolor	17.84 ± 0.238^{b}	11.70 ± 0.596^{b}
	LDN-1	26.08 ± 0.084^{e}	20.53 ± 0.948^{f}
	LDN-2	23.85 ± 0.127^{c}	17.31 ± 0.553^{e}
Means for temperature			
4 °C		23.20 ± 6.362^{B}	13.30 ± 5.153^{A}
18 °C		21.21 ± 5.789A	13.88 ± 4.804^{B}
Means for sage genotype			
Berggarten		15.46 ± 2.520^{A}	7.34 ± 0.583^{A}
Icterina		17.37 ± 0.329^{B}	9.90 ± 0.322^{B}
Purpurascens		31.14 ± 2.354^{E}	15.34 ± 1.701^{B}
Tricolor		17.73 ± 0.190^{B}	11.10 ± 0.783^{B}
LDN-1		27.26 ± 1.368^{D}	21.71 ± 1.486^{D}
LDN-2		24.29 ± 0.544^{C}	16.14 ± 1.391^{C}
Source of variation			
Temperature		***	*
Cultivar		***	***
Temperature × Cultivar		***	***

Means within a column followed by different letters (capital letters for main effects of temperature and genotype; lower case letters for interaction effect) are significantly different at $P \leq 0.05$ according to Tukey's HSD test; the level of significance ($^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; ns non-significant) for each effect (temperature, cultivar, temperature × cultivar) is also shown; ± standard deviations (SD)

in agreement with the findings of Rodríguez et al. (2015). However, chilling did not change the dry weight content in the 'Icterina' and 'Tricolor' cultivars. Comparing the genotypes, the lowest content of dry weight was determined for 'Berggarten', while a significantly higher amount of DW was found in the 'Purpurascens' sage.

The differences in the malondialdehyde content between the chilled and non-chilled sage plants were not high, but statistically significant – less MDA was found in the chilled plants in comparison to the control ones (Table 2). A higher production of MDA in the plants indicates the distinct deterioration of the membrane integrity and an increase in the MDA level is usually caused by chilling (Xu et al. 2010; Sharma et al. 2012; Lu et al. 2020), as was also found for some basil cultivars subjected to a low temperature (Kalisz et al. 2016). In the present experiment, the lower MDA content in the chilled sage does not indicate damage to the cell membrane in almost all the tested genotypes. Analysing the data for the interaction genotype × temperature, it was found that the MDA content decreased in the 'LDN-2' and 'Purpurascens' cultivars and increased in the 'LDN-1' accession, while the MDA level in the rest of the sage genotypes was not affected by the chilling. Comparing the sage genotypes, the lowest MDA content was observed

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Table 3. Total phenolic content and DPPH• radical scavenging activity in the leaves of the sage genotypes subjected to the chilling treatment

Temperature	Sage genotype	Total phenolics (mg GAE/g FM)	DPPH• (%)
4 °C	Berggarten	2.38 ± 0.046^{abc}	55.49 ± 0.517^{f}
	Icterina	2.90 ± 0.032^{ef}	53.63 ± 0.810^{ef}
	Purpurascens	2.52 ± 0.074^{bcd}	30.54 ± 1.211^{b}
	Tricolor	2.27 ± 0.066^{ab}	33.95 ± 2.187^{b}
	LDN-1	3.20 ± 0.203^{gh}	46.77 ± 2.722^{d}
	LDN-2	3.03 ± 0.044^{fg}	38.87 ± 0.306^{c}
18 °C	Berggarten	2.52 ± 0.035^{bcd}	48.53 ± 0.294^{d}
	Icterina	2.64 ± 0.027^{cde}	41.03 ± 0.530^{c}
	Purpurascens	2.15 ± 0.106^{a}	24.96 ± 1.269^{a}
	Tricolor	2.57 ± 0.145^{cd}	40.10 ± 2.322^{c}
	LDN-1	2.71 ± 0.058^{de}	46.82 ± 1.211^{d}
	LDN-2	3.39 ± 0.042^{h}	50.15 ± 0.895^{d}
Means for temperature			
4 °C		2.72 ± 0.365^{A}	43.21 ± 9.823^{B}
18 °C		2.66 ± 0.386^{A}	41.93 ± 8.746^{A}
Means for sage genotype			
Berggarten		2.45 ± 0.083^{A}	52.01 ± 3.831^{E}
Icterina		2.77 ± 0.143^{B}	47.33 ± 6.927^{D}
Purpurascens		2.34 ± 0.219^{A}	27.75 ± 3.252^{A}
Tricolor		2.42 ± 0.194^{A}	37.03 ± 3.926^{B}
LDN-1		2.96 ± 0.301^{C}	46.80 ± 1.885^{CD}
LDN-2		3.21 ± 0.199^{D}	44.51 ± 6.204^{C}
Source of variation			
Temperature		ns	*
Cultivar		***	***
Temperature × Cultivar		***	***

The means within a column followed by different letters (capital letters for the main effects of the temperature and genotype; lower case letters for the interaction effect) are significantly different at $P \leq 0.05$ according to Tukey's HSD test; the level of significance (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; ns non-significant) for each effect (temperature, cultivar, temperature × cultivar) is also shown; ± standard deviation (SD)

in the 'Berggarten' sage, while the highest level was found in the 'LDN-1' accession. These data showed that the adverse effect of the low temperature on the cell membrane stability occurred only in the 'LDN-1' sage, which was associated with non-significant changes in the antioxidant enzyme activities (Figure 1), despite an increase in the total phenolics in this sage accession due to the chilling (Table 3). It is known that phenolics cause the suppression of lipid peroxidation (Posmyk et al. 2005), but this was not confirmed for the 'LDN-1' sage.

As shown in Table 3, the chilling did not generally affect the total phenolic content in the sage plants. Phenolic compounds are among the most bioactive secondary metabolites, and serve as ROS scavengers by locating and neutralising radicals before they damage the plant cell (Gill, Tuteja 2010; Naikoo et al. 2019). A higher production of phenolics in response to low temperature stress was reported by Oh et al. (2009), Rivero et al. (2001) and Sivaci et al. (2014). However, Kalisz et al. (2016) observed an increase in the total phenolics only in lettuce leaf basil, while other tested basil genotypes did not show any significant changes in the level of these compounds. In the present study, the content of phenolic compounds decreased after the chilling treatment (by 10–12%) in the case of two sage cultivars: 'LDN-2' and 'Tricolor'. Another response to the

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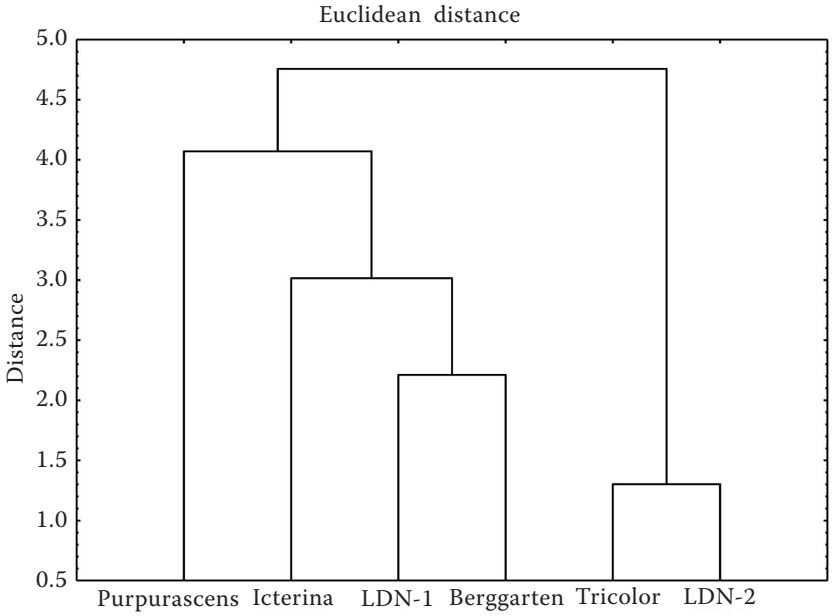

Figure 2. Dendrogram of the hierarchical cluster analysis (HCA) of all the studied sage genotypes based on the calculated differences between the chilled and non-chilled plants

chilling was found for the 'LDN-1' sage accession and the 'Purpurascens' cultivar, where the low temperature elevated the total phenolic content by 18% and 17%, respectively. Thus, the chilling had mixed effects on the level of these compounds. The highest content of the total phenolics was found in the leaves of the 'LDN-2' plants, especially in comparison to the 'Berggarten', 'Purpurascens', and 'Tricolor' genotypes. The abovementioned data indicates the importance of genotype screening to find common mechanisms in response to stress stimuli.

In contrast to the total phenolics, the DPPH• radical scavenging activity was significantly higher in the chilled sage (Table 3). The low temperature increased the DPPH• activity especially in the case of the 'Berggarten', 'Icterina', and 'Purpurascens' genotypes (by 14–31%); however, the chilling significantly decreased this activity in 'LDN-2' and 'Tricolor'. Similarly, Kalisz et al. (2016) found an increase in the DPPH• scavenging activity of chilled red basil, cinnamon basil, and lettuce leaf basil, while for three other basil cultivars, chilling did not affect the scavenging ability. Bączek-Kwinta et al. (2007) and Pohl et al. (2019) also described the genotypic diversity in the total antioxidant capacity of basil and eggplant, respectively, subjected to chilling. In the present study, the scavenging abilities of 'Berggarten' was generally the highest, while 'Purpurascens' showed the lowest level in the DPPH• scavenging activity.

There was a significant correlation between the content of the phenolic compounds in the sage plants and the antioxidant activity showed by the plants (r = 0.773, P = 0.000, n = 30). Such linear correlations have also been observed in other studies (Djeridane et al. 2006; Wojdyło et al. 2007; Oh et al. 2009), and phenolic compounds contribute to a major share of the antioxidant capacity of several plants (Wojdyło et al. 2007; Kalisz et al. 2016). However, the results obtained for 'Berggarten' (both chilled and non-chilled plants) showed a negative correlation between the total phenolic content and the DPPH• scavenging activity, and, for this reason, they were excluded from the above described analysis.

A hierarchical cluster analysis (HCA) grouped the sage genotypes into several clusters as shown in Figure 2. The moment of clustering was chosen to be halfway up the maximum distance, which was also based on the agglomeration chart. The statistical analysis showed that the 'Tricolor' and 'LDN-2' cultivars are in one basic cluster. This cluster comprised the cultivars for which the DPPH• radical scavenging activity and total phenolic content decreased the most due to the chilling. The 'Icterina' sage showed the lowest increase in the POD activity, but the highest increase in the DPPH• scavenging activity. 'Purpurascens' was characterised by the highest increase in the POD due to the chilling and had a low DPPH• scavenging activity after the low temperature treatment. The remaining genotypes, the 'LDN-1' accession and 'Berggarten' cultivar, showed a stable CAT activity and had a medium increase in the POD activity. These results confirmed the genotypic diversity in response to the chilling.

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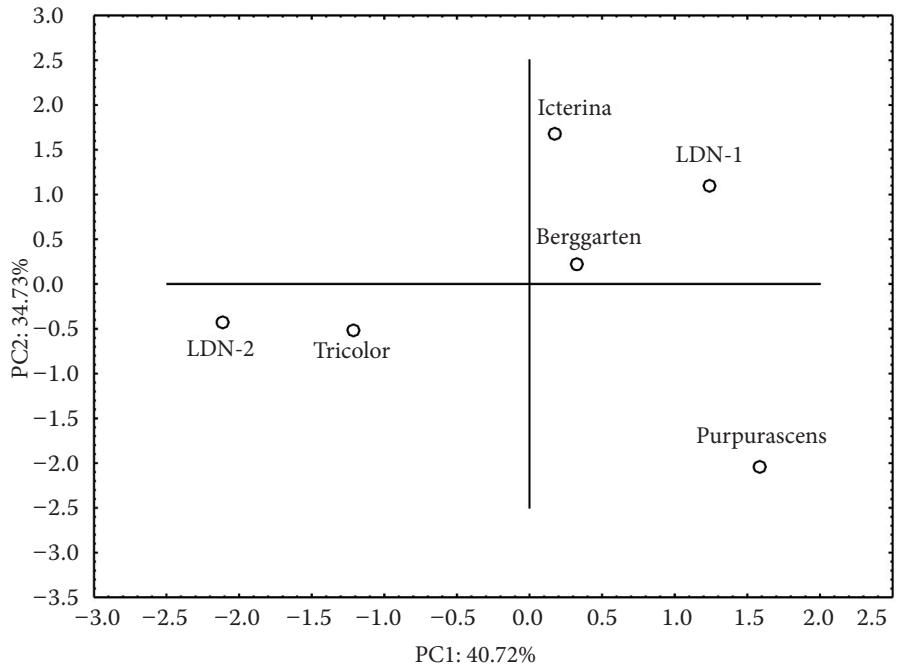

Figure 3. Principal component analysis of the sage genotypes based on the PC1 and PC2 scores

The PCA analysis reinforced the results previously observed in the hierarchical cluster analysis. The *Salvia officinalis* genotypes were located at different points in the two-dimensional space described by two PCs, principal component 1 (40.72%) and principal component 2 (34.73%) (Figure 3). One genotype ('Purpurascens') was plotted on the lower right pool of the PCA diagram. We identified another group that consisted of the 'LDN-2' and 'Tricolor' cultivars indicating that they are closely related genotypes in terms of their responses to chilling. However, the grouping of the sage genotypes in the higher right pool of the diagram is less obvious. Based on the HCA, 'Berggarten' and 'LDN-1' were grouped together, while a separate group was found for 'Icterina'. Indeed, 'Icterina' exhibited the lowest PC1 score, but the highest PC2 score among these three genotypes, and both these loadings were positive (0.125 and 1.272, respectively).

CONCLUSIONS

The exposure of sage plants to low temperature generally increased the peroxidase activity, dry weight content and DPPH• radical scavenging activity, and, at the same time, it decreased the MDA content in the leaves. The results from this study show that there is strong genotypic diversity in the response of sage to the chilling treatment. The enzymatic activity was the most pronounced in purple sage ('Purpurascens', 'Tricolor'), whereas a high DPPH• scavenging activity was observed in 'Berggarten' and 'Icterina' (silvery-blue and yellow-green leaves, respectively). The antioxidant activity and total phenolic content of the 'Purpurascens' sage (purple to silvery-green leaves) was also high, in contrast to 'Tricolor'. The sage genotypes demonstrating the strongest, but most differentiated, response were identified. The acclimation process for the 'Icterina' plants was based on the activation of the non-enzymatic antioxidants, while for 'Purpurascens' the process was based on the antioxidant enzymes (peroxidise, but not catalase). Attention should be drawn to the 'LDN-2' sage accession, for which the activity of antioxidant enzymes (POD, CAT) was similar between the chilled and non-chilled plants and the highest decrease in the DPPH• radical scavenging activity was found due to the chilling. It seems the acclimation process of 'LDN-2' plants is complete after 2 weeks of chilling.

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