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Original Scientific Paper

The antioxidant response of *Hedera helix* leaves to seasonal temperature variations

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ABSTRACT:
Seasonal variations in the environment (e.g. of temperature and light intensity) can lead to the excess production of reactive oxygen species and oxidative stress, inducing changes in the permeability of the plant cell membrane and the structure and function of cellular molecules. To address these deleterious effects, plants activate various non-enzymatic and enzymatic antioxidants. This study seeks to detect the influence of seasonal variation in *Hedera helix* (ivy) leaves, collected in Banj brdo (Banja Luka, Republika Srpska, Bosnia and Herzegovina) between December 2017 and November 2018, on oxidative (hydrogen peroxide and malondialdehyde) and antioxidant (superoxide dismutase, catalase, Class III peroxidases, and phenolic compounds) parameters. During the winter-early spring months (a temperature range of 0.7 to 5.4°C), we detected an increase in the values of all the oxidative and antioxidant parameters, whereas during the spring, summer, and autumn months (a temperature range of 15 to 25°C), the values of most of these parameters fell. However, a peak in the parameter values was detected during June and July 2018, which might be attributable to the influence of the changes in both light intensity and temperature and to the effects of intensive shoot growth. Our results highlight the importance of the antioxidant protection system of *H. helix* for its acclimation to seasonal variations in the environment, especially temperature.

Keywords:
ivy leaves, lipid peroxidation, oxidative stress, natural habitats, adaptation

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INTRODUCTION

Numerous environmental factors affect a plant's structural and biochemical properties. Temperature extremes, for example, can disrupt plant cell homeostasis (Sharma *et al*. 2012), the plant's response to such stresses varying with species, temperature interval, and exposure time. Low and high temperatures can impair both growth and yield, and also can result in the overproduction of reactive oxygen species (ROS) (Awasthi *et al*. 2015), most notably the superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and the hydroxyl radical ($^{\cdot}OH$). This group of highly reactive plant cell molecules, formed as intermediates in oxygen metabolism have two effects on plants depending exclusively on their concentration: in low/moderate concentrations, they act as secondary messengers in intracellular signalling, while in high concentrations, they have a

destructive effect on proteins, lipids, and deoxyribonucleic acid, leading to changes in the structure and function of the biomolecule (Das & Roychoudhury 2014). In response to the effects of high ROS concentrations, a plant's antioxidant enzymes and non-enzymatic antioxidants cooperate in ensuring its survival in the extreme temperatures of the natural environment. The more active a plant's antioxidant defence system, the more tolerant or resistant it is to temperature stress (Awasthi *et al.* 2015). Among the antioxidant enzymes, the specific activity of superoxide dismutase (SOD) and catalase (CAT) plays an essential role in eliminating ROS: SOD catalyses the dismutation of $O_2^{\cdot-}$ to H_2O_2, while CAT catalyses the decomposition of H_2O_2 to water (Mittler 2002; Gill & Tuteja 2010). Similarly, Class III peroxidases (PODs), belonging to a large family of multifunctional enzymes, catalyse the reduction of H_2O_2 by employing various substrates (e.g. phenolic compounds) (Passardi *et al.* 2005; Veljović Jovanović *et al.* 2018). PODs influence the regulation of H_2O_2 levels and ROS production (hydroxyl and perhydroxyl radicals) within cells and in the cell wall, vacuoles, and the apoplast (Veljović Jovanović *et al.* 2018). Phenolic compounds are non-enzymatic plant antioxidants which neutralise ROS directly, by acting as enzyme substrates or by chelating metal ions which promote the formation of ROS (Hider *et al.* 2001).

Hedera helix L. (Araliaceae) is a perennial evergreen herbaceous vine or climber, although it can be a woody shrub and also, but rarely, a tree. The natural distribution of *H. helix* is limited to Europe, North Africa and West Asia, but it has spread worldwide from Australia and New Zealand, through India, Brazil, and South Africa, to Hawaii and North America (Metcalfe 2005; Strelau *et al.* 2018). It grows successfully in a large variety of habitat types, including woodlands, forest edges, fields, hedgerows, and flood plains, as well as disturbed habitats (Metcalfe 2005; Strelau *et al.* 2018), from sea level to 1000 m a.s.l. (Reichard 2000). In Republika Srpska (part of Bosnia and Herzegovina), *H. helix* is widespread in different plant communities, but is most commonly present in woodlands and woodland edges near rivers. The optimal temperature for *H. helix* growth is 21°C (Strelau *et al.* 2018), with 10°C being sufficient for new internode growth (Strelau *et al.* 2018) and a mean above 15°C being required for sexual reproduction (Strelau *et al.* 2018). The different climate types in Republika Srpska, ranging from moderate continental (the Northern Peri-Pannonian region) and Alpine and Pannonian (affecting most of the area of Republika Srpska) to modified Mediterranean-Adriatic (mainly in the south), are the result of natural factors and the general circulation of air masses in this region (Malinović *et al.* 2018; Dragić *et al.* 2019). Cold winters and warm summers characterise the Peri-Pannonian region, with average annual air temperatures ranging from 12 to 19°C. July is the warmest month (with a mean of 21–23°C) and January is the coldest (a mean between -0.2 and -0.9°C). The region's annual temperature amplitude is high (absolute max. 41°C vs absolute min. air temperature -30°C) (Malinović *et al.* 2018; Dragić *et al.* 2019). It receives ca. 1900 sunshine hours throughout the year. *Hedera helix* is considered a semi-shade plant with 5–10% relative light flux (Ellenberg 1988) and it grows most vigorously in shaded, moist sites on heavy, fertile soils, in woodland areas (Metcalfe 2005), and, as such, is more usually exposed to low temperatures. Being an evergreen endows the plant with the following advantages: 1) a longer annual photosynthetic period; 2) lower consumption of carbon (C) for leaf renewal; 3) lower consumption of energy for the allocation of nutrients; and 4) stronger leaves, with more mechanical tissue able to withstand low temperatures (Givnish 2002). Although *H. helix* is an evergreen, seasonality significantly influences the leaves' metabolic processes. One of the putative adaptive strategies of *H. helix*, accounting for the plant's adaptation to environmental change, is its robust antioxidant protection system.

In this study, we examine the seasonal dynamics of the oxidative and antioxidant parameters of *H. helix* leaves in the plant's natural habitat in the course of one calendar year and relate them to changes in environmental factors, primarily temperature. To the best of our knowledge, the extant literature offers no reports on the changes in the antioxidant metabolism (enzymatic and non-enzymatic) of evergreen plants, including *H. helix*, during the one-year growth cycle. Our results contribute to providing a better understanding of the adaptive strategies adopted by evergreen plants to the challenges attributed to seasonal environmental changes.

MATERIALS AND METHODS

Plant material. *Hedera helix* leaves were collected from Banj brdo, Banja Luka (Republika Srpska, Bosnia and Herzegovina) between December 2017 and November 2018 (Supplementary Table 1). All the experiments were performed on the leaves of non-flowering shoots taken from five individual plants of the species *H. helix*. A mixed sample of 20–30 individual leaves collected from each plant was used for analysis. After sampling, the leaf stalk was removed and the blade was washed with distilled water, packed in aluminium bags and stored in liquid nitrogen until extraction in the laboratory. Samples were collected once a month, usually in the morning (9:30 AM) of the last Saturday of the month.

Photosynthetic pigment concentrations. The plant tissue (0.5 g) was ground with liquid nitrogen to a powder and photosynthetic pigments were extracted with 80% methanol (4 mL) containing $CaCO_3$. After centrifugation for 10 min at 10,000 rpm (Tehtnica, Centric 200R), the

Table 1. Temperature and solar insolation data for Banja Luka in 2017 and 2018 (Malinović *et al*. 2018; Dragić *et al*. 2019).

	Dec 2017	Jan 2018	Feb 2018	March 2018	April 2018	May 2018	June 2018	July 2018	Aug 2018	Sep 2018	Oct 2018	Nov 2018
Temperature, monthly mean (°C)	4.6	5.3	0.7	5.4	16.2	19.2	20.9	22.2	23.3	17.4	13.7	8
Temperature measured at the time of sampling (°C)	3.8	6.8	0.2	17.2	18.2	18	20.6	24.9	17.7	11	21.1	5.7
Minimum monthly temperature (°C)	0	0.2	-9.4	-7.2	11	16	15.6	18.7	14.4	7.3	10.3	-2.5
Maximum monthly temperature (°C)	10	10.7	12.4	16.9	21.9	24	26	26.4	26.9	21.6	22.2	15.3
Insolation (hours)	93.1	113	51	106	245.7	253	227.2	279.1	301.8	255	188.1	86.2

supernatant was used to determine the concentration of photosynthetic pigments by measuring the absorbance (Shimadzu UV-1800) of chlorophyll *a* (Chl *a*) at 666 nm, chlorophyll *b* (Chl *b*) at 653 nm, and carotenoids (Car) at 470 nm (Holm 1954). The concentrations of Chl *a*, Chl *b*, total chlorophyll, and Car are expressed in μg g_{FW}^{-1}. The ratio of Chl *a* to Chl *b* concentrations as well as the ratio of total Chl/Car concentrations were also calculated.

Extraction of malondialdehyde and H_2O_2. The plant tissue (1 g) was ground with liquid nitrogen to a powder and homogenised with 7 mL of 0.1% trichloroacetic acid (TCA). The mixtures were centrifuged for 20 min at 10,000 rpm (Tehtnica, Centric 200R) to obtain the supernatant for the determination of malondialdehyde (MDA) and H_2O_2.

Determination of MDA concentration. The samples in 0.1% TCA (0.5 mL) were mixed with 1 mL of 0.5% thiobarbituric acid dissolved in 20% TCA. The mixture was heated at 95°C for 30 min and then cooled quickly in an ice bath. The samples were centrifuged again for 10 min at 10,000 rpm (Tehtnica, Centric 200R). The supernatant was separated and its absorbance at 532 nm determined by spectrophotometry (Shimadzu UV-1800) and the value of nonspecific absorption measured at 600 nm was subtracted from it. The concentration of MDA was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} (Heath & Packer 1968).

Determination of H_2O_2 concentration. The samples in 0.1% TCA (0.5 mL) were mixed with 0.5 mL of 10 mM Na-phosphate buffer pH 6.8 and 1 mL of 1 M KI. A KI and Na-phosphate buffer pH 6.8 of the same concentrations as in the reaction mixture was used as a blank. The nonspecific absorption of the extract was determined in a blank solution containing the plant extract and Na-phosphate buffer pH 6.8. Absorbance at 390 nm was measured by spectrophotometry (Shimadzu UV-1800) and the H_2O_2 concentration was determined using a standard curve in the range of 0.01 to 0.3 M (Velikova *et al*. 2000).

Extraction of soluble proteins. For protein extraction, 0.5 g of *H. helix* leaves were ground in liquid nitrogen and homogenised with 4 mL of extraction buffer [Na-phosphate (NaPi) 0.1 M pH 6.4 containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.2% TWEEN (the commercial name for polyethylene glycol sorbitan monolaurate), and 1% polyvinylpyrrolidone (PVP)]. After centrifugation for 10 min at 10,000 rpm (Tehtnica, Centric 200R), the supernatant was separated and used to determine the protein concentration, the spectrophotometric determination of CAT and POD activities, and the separation of SOD isoforms (the modified method as described by Kukavica *et al*. 2012).

Determination of protein concentration. The protein concentration was determined using the Lowry method (Lowry *et al*. 1951). Absorbance was measured at 550 nm (Shimadzu UV-1800). To calculate the protein concentration, bovine serum albumin (BSA) was used as the standard and the protein concentration was expressed in mg g_{FW}^{-1}.

Determination of CAT activity. CAT activity was determined in a reaction mixture containing 2865 μL of 10 mM Na-phosphate buffer pH 6.8, 100 μL of supernatant, and 35 μL of 3% H_2O_2 solution. The decrease in H_2O_2 was measured as the decline in optical density at 240 nm (Shimadzu UV-1800) and the activity was calculated as μmol of H_2O_2 consumed per minute (Kato & Shimizu 1987). The extinction coefficient for H_2O_2 at the aforementioned wavelength is 40 mM^{-1} cm^{-1}.

Determination of the specific activity of POD. POD activity at 430 nm was measured by spectrophotometry (Shimadzu UV-1800) at 37°C in a reaction mixture containing 2860 μL of Na-phosphate buffer pH 6.4, 30 μL 1 M pyrogallol, and 100 μL extract. The reaction was started by adding 10 μL of 1 M H_2O_2 solution and the increase in absorbance over a one-minute period was measured (Teisseire & Guy 2000). The extinction coefficient for purpurogallin is 12 mM^{-1} cm^{-1}.

Determination of POD and SOD isoforms. Discontinuous native gel electrophoresis (BioRad Mini-PROTEAN Tetra Cell) was used to detect POD and SOD isoforms. Fifteen micrograms of protein was applied to the gel. Currents of 100 and 150 V were used to pass the samples through the concentration and separation gels, respectively. After electrophoresis, the gels were stained for POD and SOD activity. PODs were visualised by immersing the gels in 95 mL of 0.1 M Na-phosphate buffer pH 6.4 containing 5 mg of the α-chloro-naphthol dissolved in 5 mL of methanol and 100 µL of 30% H_2O_2. The SOD staining solution contained 80 mL of 0.1 M TRIS buffer [tris(hydroxymethyl)aminomethane] pH 7.8, 240 µL of 0.5 M ethylenediaminetetraacetic acid, 16 µL of tetramethylethylenediamine, 16 mg of nitroblue tetrazolium, and 4 mg of riboflavin. The gels were incubated in a specific SOD staining solution for 30 min in the dark, rinsed with distilled water, and then exposed to light for 15 min until achromatic bands appeared, in line with the method described by Beauchamp & Fridowich (1971). The isoenzyme profiles for the individual samples (1–5) were presented on the gels. The total SOD activity was obtained by adding the activity of the individual isoforms for all five *H. helix* leaf samples by means of densitometry using Image Master TotalLab TL 120 software (TotalLab 2020).

Determination of concentration of phenolic components. The concentration of phenolic compound was determined by the extraction of the plant tissue (0.5 g) with 80% methanol (4 mL). After centrifugation for 10 min at 10,000 rpm (Tehtnica, Centric 200R), the supernatant was used to determine the concentration of the total phenolics by measuring absorbance (Shimadzu UV-1800) at 724 nm using the Singleton & Rossi (1965) method. The Folin-Ciocâlteu reagent was used to determine the concentration of the phenolic compounds with gallic acid as the standard.

Detection of catechin and chlorogenic acid by the HPLC method. The methanolic extracts of *H. helix* leaves were filtered through 0.45-µm nylon microfilters and analysed using an Agilent 1260 Infinity DAD-HPLC system. Chromatographic conditions: reversed-phase column EC-C18, Poroshell-120 (4.6 × 50 mm, particle size 2.7 µm). The mobile phases were 0.1% acetic acid (solvent A) and acetonitrile (solvent B), the flow rate was 0.5 mL min^{-1}, the column temperature 25°C, and the sample injection volume 2 µL. The chromatograms were evaluated at λ = 280 nm. The following gradient was used for separation: 9% methanol (B) was released through the column isocratically with 91% solvent (A) for 0.8 min. This was then increased to 55% acetonitrile (B) for 1.5 min, changed to 40% acetonitrile (B) for 2.3 min, to 30% acetonitrile (B) for 6.8 min, and finally 40% acetonitrile (B) for 8 min. The phenolic compounds were identified by comparing the chromatographic retention times and spectra with the standards (Stojković *et al.* 2021).

Statistical analysis. The mean values of all the parameters obtained from the data for the individual samples of the species *H. helix* were used in our data processing. The research design is longitudinal, with each individual being sampled once a month in the period between December 2017 and November 2018. Three statistical procedures were performed when analysing the data:

1. Descriptive analysis of the measurement variations;
2. A comparison of the components of the average variation between the months using the paired t-test and the Wilcoxon test;
3. An analysis of the monthly correlation using Pearson's coefficient of determination.

Software R version 3.6.3 and R studio 1.2.1 were used for statistical and graphical data processing (R Core Team 2016; R Studio Team 2020).

RESULTS

Temperature and solar insolation data. The main meteorological parameters were obtained from the Hydrometeorological Institute of the Republika Srpska (HIRS) (see Table 1, Supplementary Table 2; Malinović *et al.* 2018; Dragić *et al.* 2019), while the air temperature at the sampling site was recorded once a month coinciding with the plant sampling. Solar insolation data (h) for Banja Luka were also provided by the HIRS (see Table 1). The lowest average monthly temperature in Banja Luka was 0.7°C corresponding to February 2018, while the highest was measured at 23.3°C in August 2018 (Table 1). The temperature data are presented in each of the figures below in conjunction with each of the biochemical parameters measured.

December 2017 to March 2018 were the months with the lowest number of insolation hours (below 200 h) vs April 2018 to September 2018 (above 200 h) (Table 1). The lowest number of insolation hours was recorded in January 2018 (51 h), while the highest was recorded in August 2018 (301.8 h).

The concentration of photosynthetic pigments. Between December 2017 and February 2018, the Chl *a* content (Fig. 1A) in the *H. helix* leaves increased, although this increase was not statistically significant; it then fell and remained at a low concentration until August 2018 (with a statistically significant difference noted from April–June, $p < 0.00$), before gradually increasing between September and November 2018 (September–October, $p < 0.00$). Also, a statistically significant difference ($p < 0.01$) was measured for the Chl *a* concentration between August–September. A similar annual pattern was presented by the Chl *b* content (Fig. 1B), however, the concentrations of Chl *b* were higher between April and

Table 2. Correlations between the biochemical parameters and the temperature (Pearson's coefficient of determination). Significant results are marked with an asterisk.

Parameter vs Temperature	R	p-value
MDA	-0.56	0.0580
H_2O_2	-0.5124	0.0880
CAT	-0.3475	0.2684
POD	-0.7091	0.0098**
SOD	-0.8084	0.0015**
Proteins	-0.6119	0.0345*
Phenolic compounds	-0.6405	0.0249*
Catechin	-0.2401	0.4522
Chlorogenic Acid	-0.6183	0.0321*
Chl *a*	-0.737	0.0062**
Chl *b*	-0.6761	0.0158*
Car	-0.9131	0.0000***
Total Chl	-0.7576	0.0040**

June 2018. The highest Chl *b* concentration was recorded in February 2018 coinciding with the lowest average monthly temperature and the lowest number of insolation hours (Table 1), while the lowest Chl *b* concentration was recorded in August coinciding with the highest average monthly temperature and the highest number of insolation hours (Table 1). Statistically significant differences for the Chl *b* concentrations were detected between June–July ($p < 0.01$) and July–September ($p < 0.00$). High concentrations of Car in the *H. helix* leaves were recorded in the period December 2017 to May 2018 with statistically significant differences ($p < 0.05$) for February–March and April–May (Fig. 1C), while the lowest concentrations were recorded in the period from June to August 2018 ($p < 0.01$ for July–August). From September 2018 onwards, the temperatures fell accompanied by a new increase in Car concentrations (Fig. 1C). Our results also show that the total Chl (Fig. 1D), as well as the Chl *a*/*b* ratio (Fig. 1E), were generally higher in the period between December 2017 and May 2018. Finally, Fig. 1F shows that the highest total Chl/Car ratio was recorded between June and August 2018 but is not statistically significant. Statistically significant differences for Chl/Car were detected between December–January and May–June ($p < 0.00$).

Oxidative parameters

Concentrations of MDA and H_2O_2. Figure 2 shows the annual changes in the MDA (Fig. 2A) and H_2O_2 concentrations (Fig. 2B) in the *H. helix* leaves. The highest MDA concentration was recorded in March 2018 (0.0157 µmol g_{FW}^{-1}) and the lowest in April 2018 (0.0015 µmol g_{FW}^{-1}). The most marked increases in MDA concentrations were observed in the periods from December 2017 to March 2018 (winter/early spring) and from September to November 2018 (late autumn). Statistically significant differences for MDA concentrations were detected between February–May and June–July ($p < 0.00$); January–February, May–June ($p < 0.01$); December–January, August–September ($p < 0.05$). The highest H_2O_2 concentration was also recorded in March 2018 (0.5637 µmol g_{FW}^{-1}) and the lowest in June 2018 (0.0727 µmol g_{FW}^{-1}) (Fig. 2B). For H_2O_2 concentrations, statistically significant differences were detected between December–January and February–April ($p < 0.01$) and between the June–July samples ($p < 0.00$).

Antioxidant parameters

Soluble protein concentration. Our results show that the lowest concentration of soluble proteins in the *H. helix* leaves was recorded in May 2018 (3.37 µmol g_{FW}^{-1}), while the highest concentration was in February 2018 when it reached a value of 13.67 µmol g_{FW}^{-1} (Fig. 3A). The greatest increase in protein concentration was measured during the colder months (December 2017–April 2018 with $p < 0.00$ between January–April and September–November 2018); however, the protein concentration in the samples also increased during the summer (June–August 2018, with $p < 0.01$ between June–July).

Specific SOD activity. The highest total SOD activity was recorded in the period from December 2017 to April 2018, with statistically significant differences between January–February and April–May ($p < 0.00$), the highest value being reached in January (Fig. 3B). The activity fell in May and did not change significantly until August 2018 (August–September, $p < 0.00$). The lowest total SOD activity was measured in June 2018. In September 2018, SOD was reactivated in the *H. helix* leaves, a process which continued through the late autumn and winter months (October–November, $p < 0.00$).

Specific CAT activity. Higher CAT activity was recorded during the winter months (December–March with $p < 0.05$ for December–January) than in the spring (April and May). Statistically significant differences ($p < 0.01$) in CAT activities were detected between January–February and March–April. As the temperatures peaked (June–August), CAT activity increased, but this increase was not statistically significant. However, after a marked decrease in September, an increase in activity was recorded in October (September–October, $p < 0.01$) and November (Fig. 3C). A maximum CAT activity of 0.0274 µmol mg_{prot}^{-1} min^{-1} was measured in July 2018, while a minimum of 0.0035 µmol mg_{prot}^{-1} min^{-1} was recorded in May 2018.

Specific POD activity. An increase in POD activity (Fig. 3D) was recorded between December 2017 (max. 0.7064 µmol mg_{prot}^{-1} min^{-1}) and March 2018. In April, May

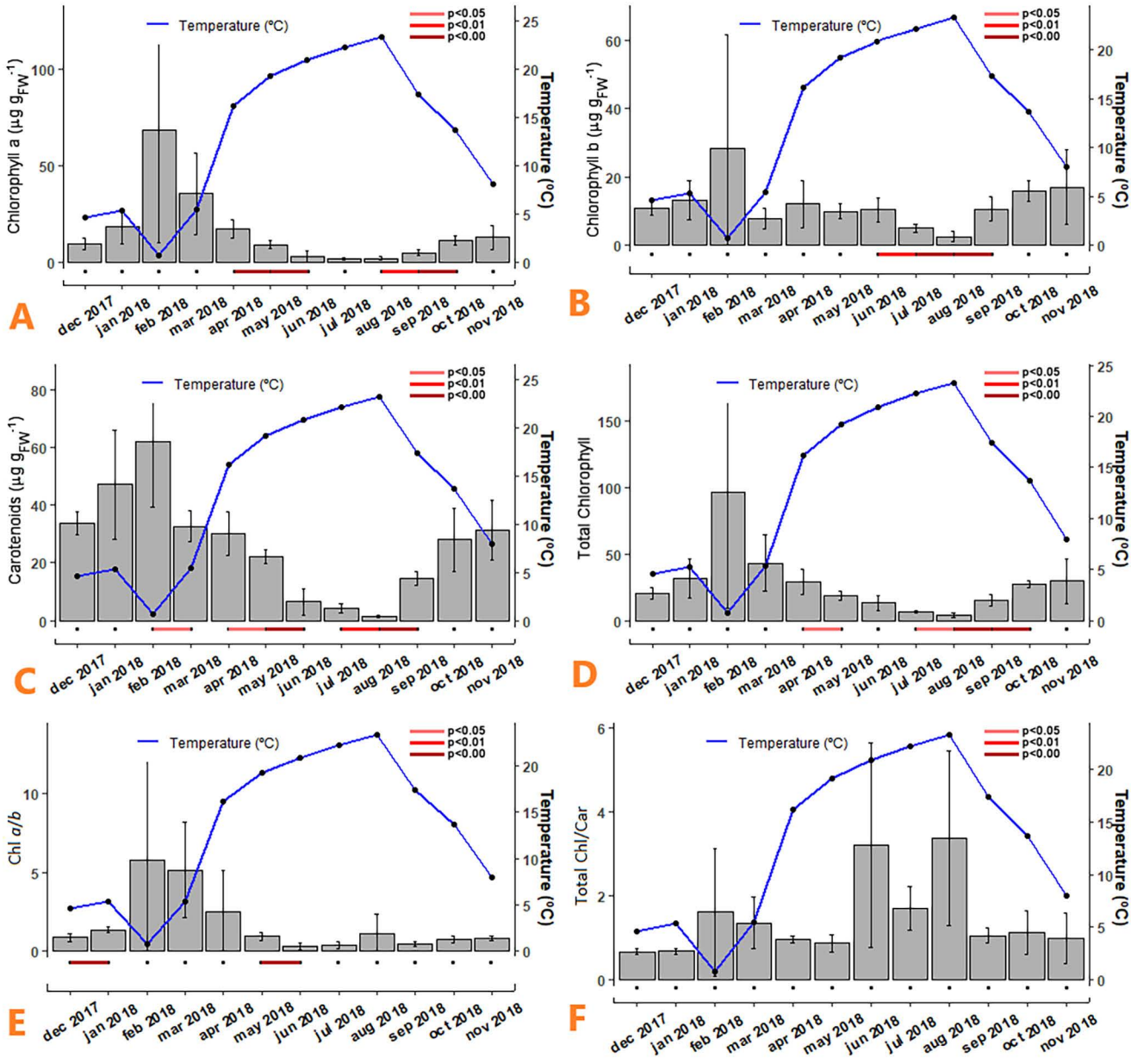

Fig. 1. Changes in the concentrations of chlorophyll *a* (A), chlorophyll *b* (B), and carotenoids (C), and changes in total chlorophyll content (D), the Chl *a*/*b* ratio (E), and the total Chl/Car ratio (F) in the *H. helix* leaf samples between December 2017 and November 2018. The temperatures in this period are presented as average monthly temperatures and given in °C. Statistically significant values are underlined in red.

(min. 0.0421 µmol mg_{prot}^{-1} min^{-1}) and June of that year, as well as in August and September, the POD activity values were low (Fig. 3D), while a spike in activity was recorded in July 2018 (July–August, $p < 0.05$).

SOD and POD isoenzyme profiles. Fig. 4 shows the SOD isoforms of the *H. helix* leaves detected on the native gel for each of the twelve months. The maximum number of SOD isoforms (six) was observed in the period between December 2017 and February 2018 (Fig. 4). In March 2018, SOD4, SOD5 and SOD6 isoforms were not detected on the gels, but a new isoform, SOD1', appeared, characteristic of March and April 2018 (Supplementary Table 3).

The POD isoforms of the *H. helix* leaves detected on the native gel are presented in Fig. 5. Four POD isoforms, labelled POD1–POD4 (Supplementary Table 4), were detected in most samples while POD1 and POD3 were not detected in August and February 2018, respectively, and POD4 was not detected between February and April 2018 (Supplementary Table 4). However, the isoform labelled POD1' was induced in most samples in March and April.

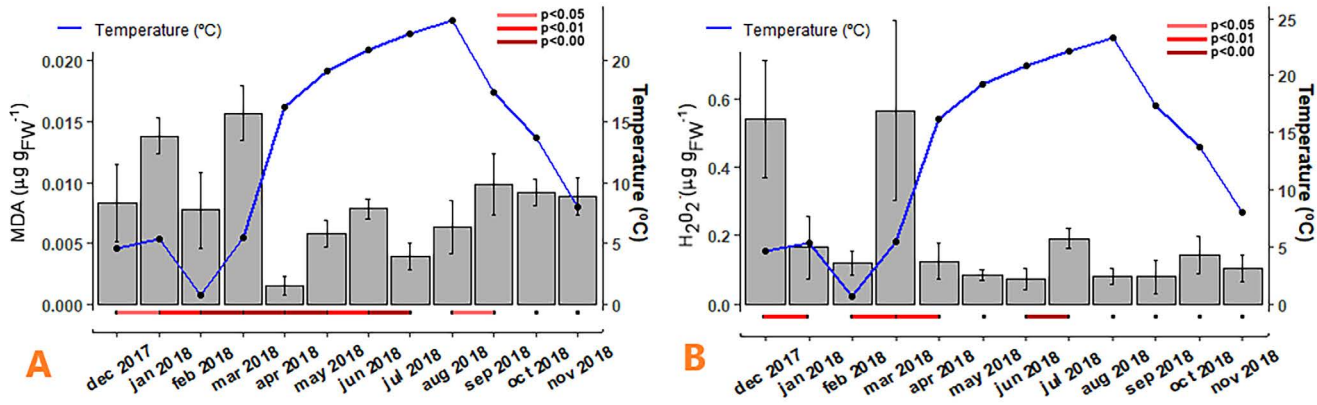

Fig. 2. Changes in the concentration of the oxidative parameters: MDA (A) and H_2O_2 (B) in the *H. helix* leaf samples between December 2017 and November 2018. The temperatures in this period are presented as average monthly temperatures and given in °C. Statistically significant values are underlined in red.

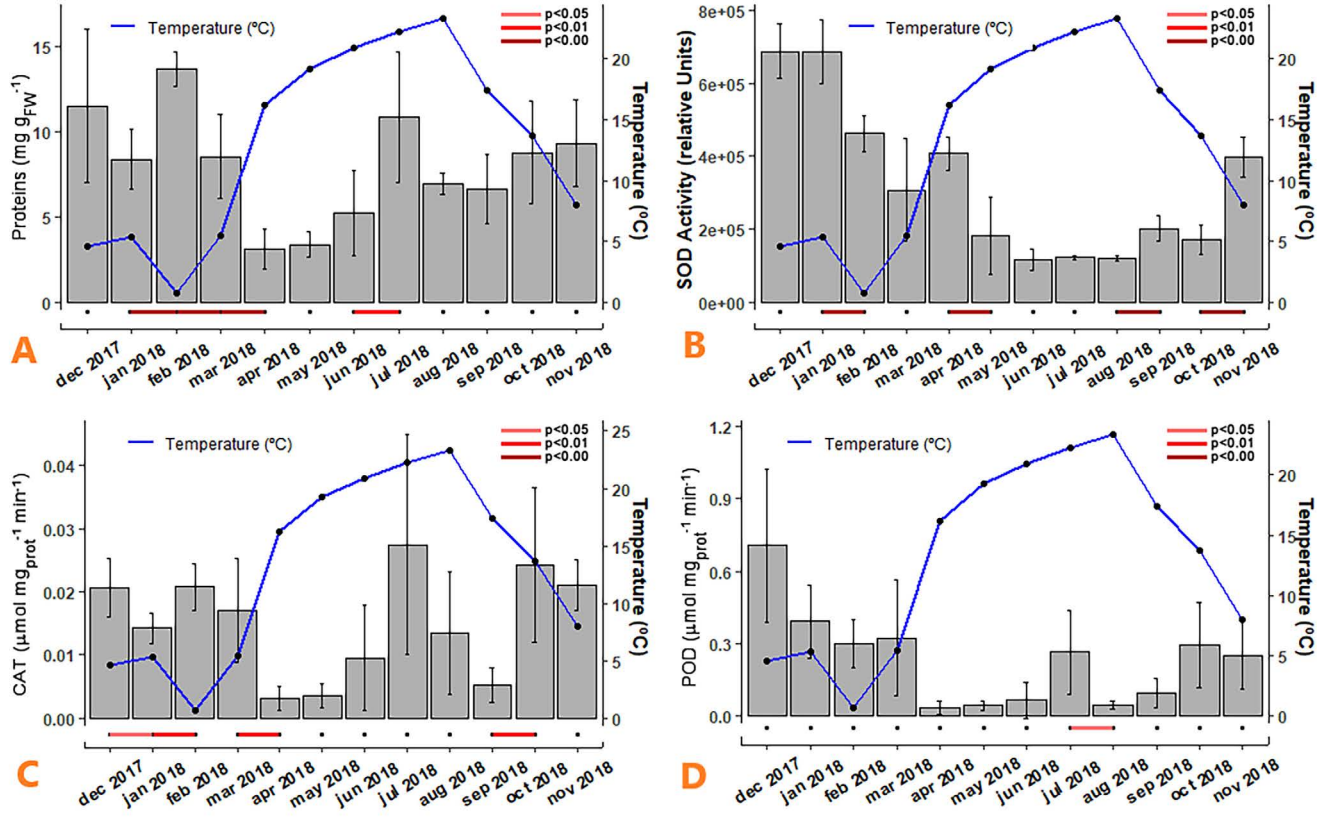

Fig. 3. Changes in the concentration of the total proteins (A), SOD activity (B), CAT activity (C), and POD activity (D) in the *H. helix* leaf samples between December 2017 and November 2018. The temperatures in this period are presented as average monthly temperatures and given in °C. Statistically significant values are underlined in red.

Non-enzymatic antioxidants

The concentration of phenolic compounds, catechin and chlorogenic acid. The highest concentration of phenolic compounds in the *H. helix* leaves was recorded in December 2017 (62.74 mg g_{FW}^{-1}) and the lowest in July 2018 (11.60 mg g_{FW}^{-1}) (Fig. 6A). A statistically significant difference ($p < 0.00$) for the catechin and chlorogenic acid concentrations was detected between March–April, while for the phenolic compounds statistically significant differences ($p < 0.01$) were found between January–February and April–May. Notable concentrations of catechin and chlorogenic acid were detected by HPLC

Table 3. The monthly correlations between the parameters (Pearson's coefficient of determination). Significant results are in bold.

Month	Independent variable: H_2O_2				Independent variable: phenolic compounds	Temp	
	Dependent variable				Dependent variable	min	max
	CAT	POD	Phenolic compounds	MDA	POD		
Dec-17	0.5585	0.2954	0.1613	0.0000	0.4815	0	10
Jan-18	0.0000	0.0000	0.1190	0.0816	***0.9795***	0.2	10.7
Feb-18	0.0000	***0.7491***	0.0000	0.0000	0.0000	-9.4	12.4
Mar-18	0.0000	0.0000	0.0000	0.0000	***0.7872***	-7.2	16.9
Apr-18	0.0000	0.0911	***0.6992***	0.4423	0.1622	11	21.9
May-18	0.4361	0.0000	0.0000	0.1349	***0.8681***	16	24
Jun-18	0.0000	0.1420	0.0000	0.3425	0.4761	15.6	26
Jul-18	0.0353	0.3496	0.5381	0.0672	**0.7633**	18.7	26.4
Aug-18	0.0000	0.0000	0.0000	0.0000	0.2505	14.4	26.9
Sep-18	0.5691	0.0000	***0.6357***	0.0000	0.0000	7.3	21.6
Oct-18	0.0000	0.0330	0.0000	0.5817	***0.9900***	10.3	22.2
Nov-18	0.4012	***0.8511***	0.0000	0.0000	0.1295	-2.5	15.3

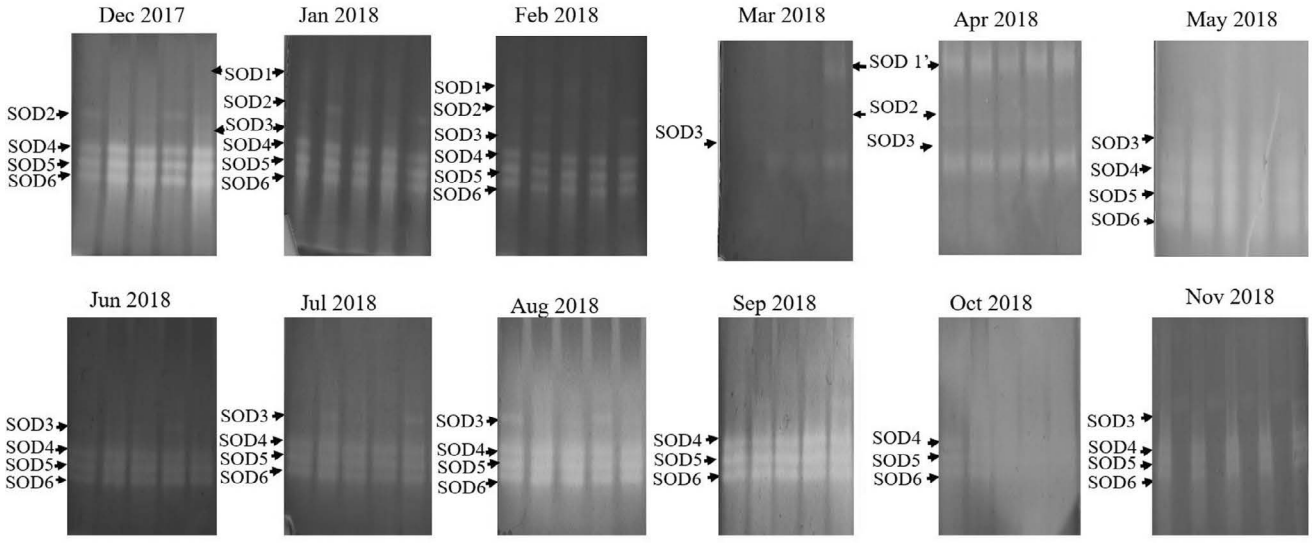

Fig. 4. Native gels with separated SOD isoforms for the period December 2017–November 2018 in the *H. helix* leaf samples. The arrows indicate SOD isoforms.

analysis in all the leaf samples (Figs. 6B & C, respectively), the lowest concentrations being recorded in April and the highest in October 2018. Additionally, naringenin was detected in sample S1–3 and quercetin was detected in sample S4–3 (not shown in the figures).

Correlations between the temperature and the biochemical parameters and photosynthetic pigments. The Pearson's correlation coefficient values for all the biochemical parameters and concentrations of photosynthetic pigments with temperature were negative (Table 2). Statistically significant strong negative correlations were shown between the activities of SOD and POD ($p < 0.01$), as well as the concentrations of Car ($p < 0.00$), Chl *a* and total Chl ($p < 0.01$) and temperature. The negative correlations indicate that increasing temperature leads to a decrease in SOD and POD activity, as well as a drop in the concentration of photosynthetic pigments.

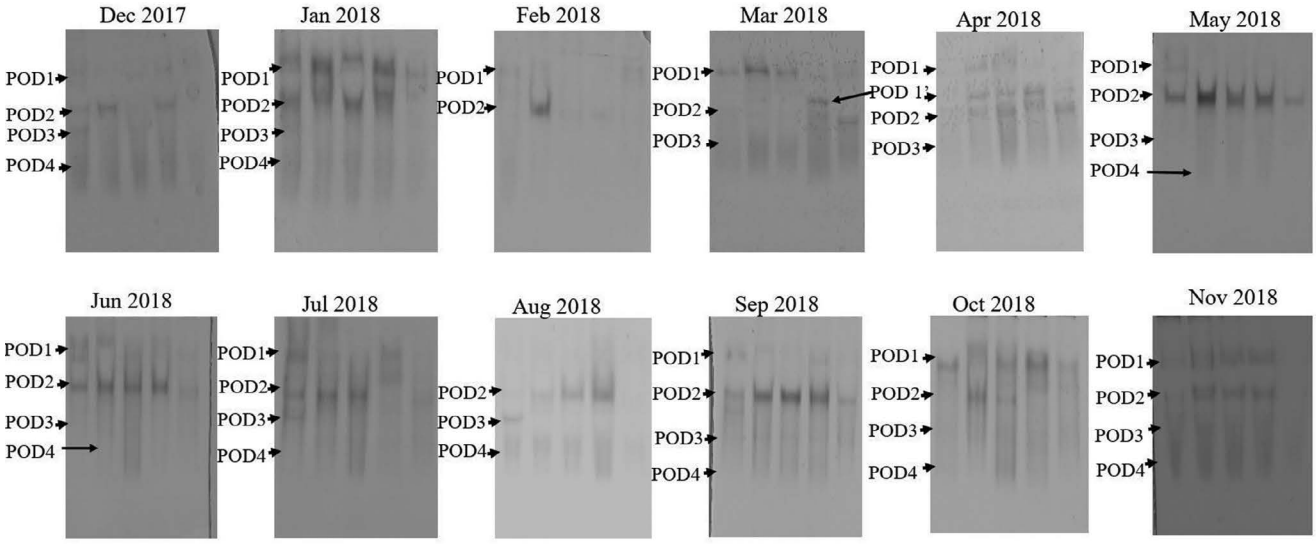

Fig. 5. Native gels with separated POD isoforms for the period December 2017–November 2018 in the *H. helix* leaf samples. The arrows indicate POD isoforms.

DISCUSSION

Hedera helix grows in the moderate continental climate of the Northern Peri-Pannonian region of Republika Srpska, characterised by moderately cold winters and warm summers and an average annual air temperature ranging between 12 and 19°C. The number of snow days in 2017 and 2018 was 48 and 52, respectively, while frost was recorded on 72 and 71 days in these two years, respectively (HIRS, see Supplementary Table 2).

We monitored the changes in the concentration of photosynthetic pigments and in the oxidative and antioxidant parameters of the *H. helix* leaves during the one-year growth cycle in the plant's natural habitat. The leaves of *H. helix* go through several stages of development: leaf initiation (March–April–May), intensive shoot growth (June–July–August), transition to deep dormancy (September–October), deep dormancy (November–December), and forced dormancy (January–February). They are also exposed to the complex influence of abiotic (low/high temperatures, high/low light intensity, frost, snow, and mechanical injuries) and biotic (pathogens and herbivores) stress during the one-year growth cycle which increases the concentration of ROS, thus resulting in oxidative stress. It was previously shown that exposure to various types of stress increases the concentration of ROS and consequently leads to oxidative stress (Hasanuzzaman *et al*. 2020). In addition, stress leads to the disruption of redox homeostasis in the plant cell, the maintenance of which is dependent on the antioxidant protection system (Hasanuzzaman *et al*. 2020).

Photosynthetic pigment concentration is a good indicator of the efficiency of photosynthesis. Here, the increase in the total Chl concentration detected in winter (Fig. 1D) is indicative of the selection of those traits which might compensate for low light intensity and facilitate a more efficient capture of solar radiation in *H. helix* leaves on winter days with favourable daily temperatures (Table 1). During the leafing period, the total Chl concentration decreased (statistically significant in April–May; July–August) with increasing temperature (Fig. 1D). However, the increase in Chl *b* and the fall in the Chl *a/b* ratio between June and July (Fig. 1B) is probably attributable to the low light intensity of the shaded woodland area (Fig. 1D) (Zhang *et al*. 2016; Hallik *et al*. 2017). Between May and August, this increase in the concentration of Chl *b* (which acts as an antenna pigment capturing and transferring high-energy electrons to Chl *a*) in relation to Chl *a* (Figs. 1B & D) is likely to contribute to quantum efficiency.

Car concentrations were the highest in the period when the temperatures were the lowest (winter–spring) (Fig. 1C). In *Pinus contorta* Loudon needles, an increase in the Car/total Chl ratio was described during frost hardening, attributable, it is argued, to the role of Car in the non-photochemical dissipation of absorbed energy, which facilitates acclimation to low temperatures (Öquist & Huner 2003). Moreover, Car are antioxidants which can quench triplet Chl and directly prevent the formation of $^1O_2$ in conditions when plants are exposed to abiotic stress (Frank & Brudvig 2004; Sarker & Oba 2020). An increase in Car concentrations may also be associated with the regulation of membrane fluidity at low temperatures as has been shown only experimentally *in vitro* (in liposomes) and *in vivo* for *Staphylococcus xylosus* cells (Gruszecki & Strzałka 2005; Seel *et al*. 2020). At low temperatures, Car synthesis and the

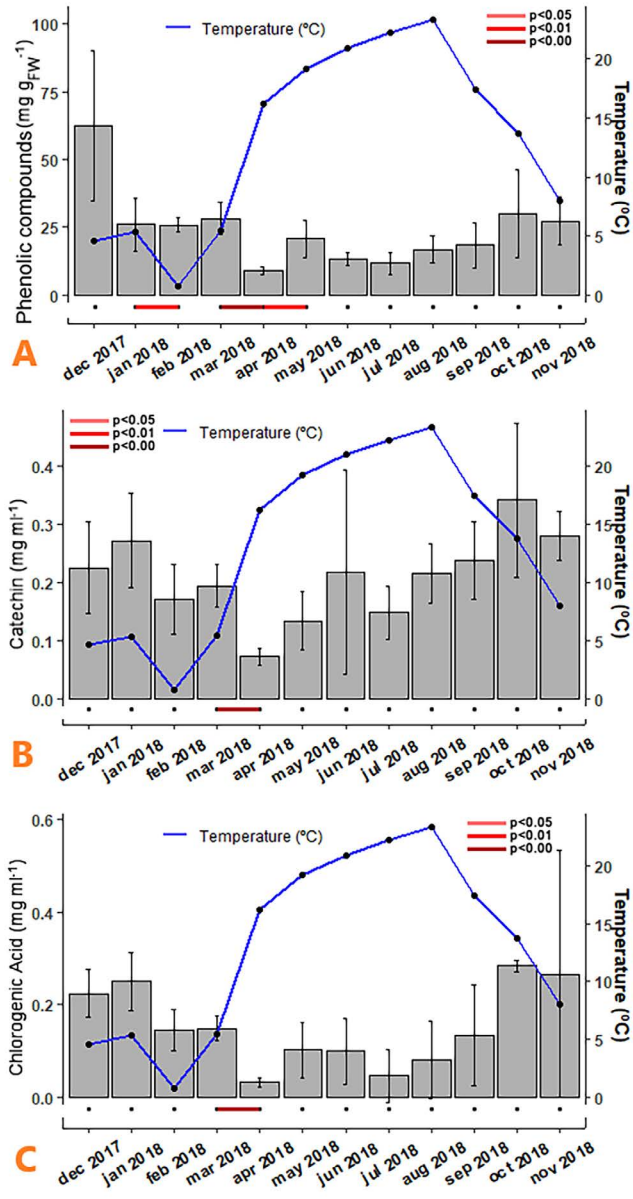

Fig. 6. Changes in the concentration of phenolic compounds (A), catechins (B) and chlorogenic acid (C) in the *H. helix* leaf samples between December 2017 and November 2018. The temperatures in this period are presented as average monthly temperatures and given in °C. Statistically significant values are underlined in red.

incorporation of these pigments into the membrane increases as does the resistance of the cells to freeze-thaw stress, as was shown for *Staphylococcus xylosus* cells (Seel *et al.* 2020). The increase in Car concentrations coincided with the coldest months, in association with the antioxidant enzyme activity of SOD, CAT, and POD, indicating that the leaves of *H. helix* are most exposed to stress in this period.

Exposure to mild frost (during periods of several weeks) affects the acquisition of resistance to severe frost. Low temperatures resulting in freezing promote acclimation to frost and temperatures down to about -12°C. Mild frost exposure extends the plant's endurance to -16°C, while exposure to -10°C induces tolerance to temperatures between -20 and -24°C (Bauer & Kofler 1987). These freeze-thaw cycles in plants serve as a natural signal to individuals to adapt to cold (Chen *et al.* 2006). In *H. helix*, this stress tolerance might be accompanied by the increased activity of one or more antioxidant enzymes (Chen *et al.* 2006). One of the consequences of exposure to freezing stress may be increased damage to cell membranes attributable to lipid peroxidation (Taulavuori *et al.* 2010). The intensity of lipid peroxidation and the influence of seasonal changes on this intensity were measured by recording concentrations of MDA, one of the end products of polyunsaturated fatty acid peroxidation in the cell (Hasanuzzaman *et al.* 2013). Thus, the increased levels of MDA from December through to March 2018 could be an indicator of the occurrence of oxidative stress in the *H. helix* leaves due to the cold (Table 1, Fig. 2A). In the leaves of evergreen *Iris hexagona* Walter, low temperatures led to a significant increase in MDA concentration in January, followed by a decrease in February, before they rose again in March (Shao *et al.* 2022). Our results in winter and early spring are consistent with those obtained for *I. hexagona* and point to the influence of low temperatures on the plant's cell membranes. An increase in the intensity of lipid peroxidation after short-term exposure to low temperatures (-5°C) was also shown in the leaves of two olive cultivars: *Olea europaea* L. cv. Leccino and cv. Oblica (Pfeiffer *et al.* 2013). The subsequent increase in the MDA content in May–June 2018 and through the autumn months might be a consequence of changes in temperature/light intensity (Table 1, Fig. 2A).

Depending on its concentration in the plant cell, H_2O_2 can be either beneficial or detrimental to plant biomolecules. At low intracellular concentrations, H_2O_2 acts as a regulatory signal for essential physiological processes, including senescence (Peng *et al.* 2005), photorespiration and photosynthesis (Noctor 2002), stomatal movement (Bright *et al.* 2006), cell cycle and growth and development (Tanou *et al.* 2009a, b). Here, significantly higher H_2O_2 concentrations were recorded in December 2017 and March 2018 than those measured in the two intervening months (Fig. 3C). This winter increase in H_2O_2 concentrations may be attributable to the low temperatures and frost in February and March 2018 or to the snowfall at the end of February 2018 and the possible mechanical injuries suffered by the leaves which came into contact with snow and ice crystals (Charrier *et al.* 2015; Wang *et al.* 2016). Although antioxidant enzyme (SOD, CAT, and POD) activity also increased in December and March (Fig. 3), it appears that the concentrations of H_2O_2 in the plant cells exceeded the capacity of the enzymes to remove it. After exposure to freezing (-5°C) for 48 h, the H_2O_2 concentration increased in the

leaves of two olive cultivars (*O. europaea* cv. Leccino and cv. Oblica) (Pfeiffer *et al.* 2013). Among its other roles in the cell, in the Fenton reaction with $Fe^{+2/+3}$ and $Cu^{+1/+2}$, H_2O_2 can generate a hydroxyl radical, which is one of the main initiators of lipid peroxidation (Halliwell 2006). We did not a correlation between the H_2O_2 and MDA concentrations during the study period (Table 3), which suggests that other types of ROS (e.g. $O_2^{\cdot-}$ and $^1O_2$) contribute to lipid peroxidation (Das & Roychoudhury 2014).

The protein accumulation in the *H. helix* leaves (Fig. 3A) was accompanied by an increase in the activity of the enzymes involved in antioxidant metabolism (Figs. 3B–D). This increase in protein concentration in the winter may be due to the increased synthesis of proteins which protect the plant from freezing and membrane damage (cold acclimation proteins) (Artlip *et al.* 1997; Öquist & Huner 2003). Protein content seasonality was measured in *Picea omorika* (Pančić) Purk. needles, although in this instance the lowest concentrations were recorded in the summer (Bogdanović *et al.* 2007). This suggests that differences in protein content may be species specific as well as the result of the intensity/duration of environmental factors at the time of sampling.

Due to its ability to neutralize the reactivity of $O_2^{\cdot-}$ overproduced under stress, high SOD activity is associated with stress tolerance in plants (Bowler *et al.* 1992). Chilling stress has been shown to have various effects on SOD activity: the onset of chilling may suppress it, and long-term chilling can activate it (Xu *et al.* 2010), while, in some instances, chilling may have no effect on SOD activity at all (Lukatkin 2002). Long-term chilling increased SOD activity (Fig. 3B), suggesting that low temperatures and/or the influence of high light intensity might explain *H. helix* resistance to stress. The fall in SOD activity in summer may be attributed to the fact that the *H. helix* leaves were shielded from the direct effects of sunlight and UV radiation by the canopy of deciduous trees (Hernandez *et al.* 2006; Cruces *et al.* 2016). One of the main sources of $O_2^{\cdot-}$ in plant cells is the electron transport chain in chloroplasts (Dreyer & Dietz 2018). Here, the increase in light intensity may have led to the formation of $O_2^{\cdot-}$, which might have induced SOD activity (Fig. 4). The increased light intensity of March and April (Table 1) may also have been the cause of the appearance of a new SOD (SOD1') isoform in the *H. helix* leaves (Table 2, Fig. 4). Likewise, leaf initiation and the intensification of metabolic processes in these two months may also have induced this new isoform. SOD isoforms 3 to 6 are present during most of the growth phases of *H. helix* leaves and, hence, during various periods of environmental change; thus, these isoforms might be labelled as the "housekeepers" of redox homeostasis in *H. helix* leaves. The SOD1 and SOD2 isoforms appear in the leaf dormancy period during low temperatures (December–February), suggesting they might be involved in protecting cells from ROS during freezing, while the induction of SOD2 can also be related to the leaf initiation period characterised by changes in light intensity (March–April). Our results indicate that different phases of growth and development, as well as changes in environmental factors (e.g. temperature), have an effect on the induction/inhibition of individual SOD isoforms. Bogdanović *et al.* (2007) showed that the seasons had a significant impact on the total SOD activity in the needles of the evergreen tree *P. omorika*. These authors also found SOD activity to be the highest in autumn and winter and the lowest in summer and that the isoenzyme profile of SOD changes with the seasons. Similarly, in a study extending from June 1988 through January 1991, Anderson *et al.* (1992) reported higher SOD activity in *Pinus strobus* L. needles in winter than in summer.

In order to scavenge H_2O_2, CAT activity increases, contributing significantly to stress tolerance (Almeselmani *et al.* 2006, 2009). Three peaks in CAT activity were recorded in the leaves of *H. helix*: December–March, June–August and October–November (Fig. 3C). These increases may have been influenced by metabolic processes as well as by the increased influence of environmental factors: December–February (low temperatures), March (low temperatures/leaf initiation), June–July–August (intensive shoot growth/light and temperature intensity), October–November (dormancy period/low temperatures and fall in insolation) (Fig. 4). The influence of the seasons on CAT activity was demonstrated in *P. omorika* needles, with the greatest activity being recorded in the summer (Bogdanović *et al.* 2007). CAT is an extremely efficient enzyme for removing H_2O_2; however, the K_m for H_2O_2 is very high, e.g. 40–600 mM (Chelikani *et al.* 2004; Mhamdi *et al.* 2010). Here, our correlation analysis indicates that the CAT activity did not significantly affect the H_2O_2 concentration (Table 3), suggesting that the H_2O_2 concentrations in the *H. helix* leaves were lower than the K_m for CAT (except in December and March) and, moreover, that other protection mechanisms (the ascorbate-glutathion cycle and ascorbate peroxidases) were involved in H_2O_2 removal.

Among the many roles that PODs play in cells, one of the main ones is the removal of H_2O_2 under conditions of physiological stress (Bania & Mahanta 2012). Increased diurnal temperature differences with the shortening of the day, the presence of frost in the morning and late evening in the autumn, and the snowfall in late February 2018 increased the POD activity in *H. helix* in the autumn–winter period (Fig. 3D). Chilling stress has been shown to induce POD activity in the non-evergreen plants of *Nicotiana tabacum* L. seedlings and *Avena nuda* L. (Xu *et al.* 2010; Liu *et al.* 2013), however, Bogdanović *et al.* (2007) reported that the seasons had no significant effect on the total POD activity in *P. omorika* needles. Nevertheless, the influence of snow cover on in-

creased POD activity was noted in the desert moss *Syntrichia caninervis* Mitt. in different microhabitats (Yin & Zhang 2016). However, few studies have examined the influence of snow on plants' POD activity. The significant increase in POD activity in July might be related to the intensive growth of shoots (June–July) because, in addition to their antioxidant role, PODs are also involved in growth and development processes (Syros *et al*. 2005; Veljović-Jovanović *et al*. 2018).

A large number of POD isoforms involved in various cellular processes (including lignification and auxin synthesis) and responses to abiotic and biotic stress have been detected in plant cells, although which specific isoform is associated with which process remains unclear. Here, POD isoforms 1 to 3 were present in the *H. helix* leaves throughout the year and, as such, can be labelled as "housekeepers" in the plant's development processes and responses to changes in the environment (Table 2, Fig. 5). The induction of the POD1' isoform (March–April) may be associated with the low temperature/initiation of leaves. The induction or inhibition of POD isoforms might be indicative of the specific roles of individual POD isoforms in adaptations to changes in temperature and/or light intensity. The expression of different POD isoforms during development and under the influence of light intensity/temperature was reported in the seedlings of the evergreen subshrub *Ebenus cretica* L. (Syros *et al*. 2005) and in the needles of *P. omorika* (Bogdanović *et al*. 2007). In *Picea* species, 15 to 20 different POD isoenzymes were detected by isoelectric focusing in the course of the one-year growth cycle (Kishchenko 2019). The author showed that these POD isoforms are characteristic of either the species' growth or dormant periods and that different isoforms are involved in the response to different types of stress. Changes in isozyme patterns can provide plants with homeostasis maintenance and tolerance to various factors in the environment (Kishchenko 2019).

Although the concentration of phenolic compounds increased in the colder months (December 2017–March 2018 and September 2018–November 2018), these changes were not statistically significant (Fig. 6A). Phenolic compounds are reported to act in the H_2O_2-scavenging system in the presence of ascorbic acid and PODs (Ferreres *et al*. 2011). A positive correlation between the concentration of phenolic compounds and POD activity was found for some months during the growth of *H. helix*, indicating that they might act jointly in the removal of ROS (Table 3). Moreover, it appears that phenolic compounds may act as antioxidants independent of POD, given that a positive correlation was observed between the concentration of phenolic components and that of H_2O_2 in April 2018 and September 2018 (Table 3).

In seasons in which the influence of frost, snow and low temperatures was clearly evident, the concentration of catechins (Fig. 6B) increased, although this increase was not statistically significant in relation to the period of higher temperatures when these factors were not present. Catechins act as antioxidants in plants by removing ROS directly, by acting as metal ion chelators, and by regulating antioxidant enzyme activities (Bernatoniene & Kopustinskiene 2018). Through these mechanisms, catechins appear to protect *H. helix* leaves from the influence of low temperatures and low light intensity. Another effective antioxidant which can protect plants against oxidative stress by enhancing their antioxidant capacity is chlorogenic acid (Grace *et al*. 1998; Mei *et al*. 2020). Exogenous chlorogenic acid was shown to effectively reduce membrane damage and lipid oxidation and to stimulate the activity of antioxidant enzymes, including PODs, CAT and polyphenol oxidase (Mei *et al*. 2020). According to recent research conducted on *H. helix* samples collected in central and southern parts of Europe, chlorogenic acid and 3,5-dicaffeoylquinic acids provide up to 80% of the plant's antioxidant activity (Bezruk *et al*. 2020). In our study, although the chlorogenic acid concentrations in the *H. helix* leaves increased in late autumn and winter, this increase was not statistically significant in relation to the late summer values (Fig. 6C).

CONCLUSION

To the best of our knowledge, the changes in the oxidative and antioxidant parameters of the leaves of the evergreen *H. helix* during a one-year growth cycle have not been reported previously. We found that in the period marked by the lowest temperatures, i.e. from December 2017 to March 2018 (0.7 to 5.4°C), the concentration of oxidative parameters (MDA and H_2O_2) increased, coinciding with the activation of all the plant's antioxidant defence parameters (SOD, POD, CAT, phenolic compounds, and carotenoids). It is apparent that *H. helix* leaves face their greatest challenges during periods of low temperature and that increased antioxidant activity is one way of adapting to these cold conditions which is also indicated by Pearson's coefficients of correlation between the temperature and biochemical parameters. The plant's antioxidant parameters may be bioindicators of changes in ambient temperature which is of particular relevance now that global climate change has acquired such relevance.

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REZIME

Botanica SERBICA

Antioksidativni odgovor listova vrste *Hedera helix* na sezonske temperaturne varijacije

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Sezonske varijacije u životnoj sredini (npr. temperature i intenziteta svetlosti) mogu dovesti do povećanog stvaranja reaktivnih kiseoničnih vrsta i oksidativnog stresa izazivajući promene u fluidnosti membrana, te strukturi i funkciji ćelijskih biomolekula. Kako bi se zaštitile od štetnih uticaja oksidativnog stresa, biljke poseduju različite neenzimske i enzimske antioksidanse. Ovo istraživanje nastoji da uoči uticaj sezonskih varijacija na listove vrste *Hedera helix* (bršljan) sakupljane na lokalitetu Banj brdo (Banja Luka, Republika Srpska, Bosna i Hercegovina) od decembra 2017. do novembra 2018. godine, na oksidativne (vodonik peroksid, malondialdehid) i antioksidativne (superoksid dismutaza, katalaza, peroksidaza i fenolna jedinjenja) parametre. Tokom zimsko-rano prolećnih meseci (temperaturni opseg 0.7–5.4°C) primećeno je povećanje vrednosti svih oksidativnih i antioksidativnih parametara, dok su tokom prolećnih, letnjih i jesenjih meseci (temperaturni opseg 15–25°C) vrednosti većine ovih parametara snižene. Međutim, maksimum vrednosti merenih parametara zabeležen tokom juna i jula 2018. godine mogao bi se pripisati uticaju promena svetlosti/ temperatura i intenzivnom rastu izdanaka. Naši rezultati naglašavaju važnost antioksidativnog sistema zaštite kod vrste *H. helix* za njegovo prilagođavanje na sezonske varijacije u životnoj sredini, posebno varijacije u temperaturi.

Ključne reči: listovi bršljana, lipidna peroksidacija, oksidativni stres, prirodno stanište, adaptacija