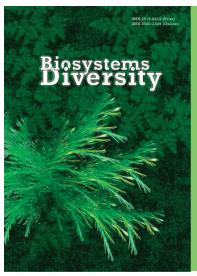

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# The influence of *Cameraria ohridella* (Lepidoptera, Gracillariidae) on the activity of the enzymatic antioxidant system of protection of the assimilating organs of *Aesculus hippocastanum* in an urbogenic environment

L. V. Shupranova, K. K. Holoborodko, O. V. Seliutina, O. Y. Pakhomov

*Oles Honchar Dnipro National University, Dnipro, Ukraine*

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*Oles Honchar Dnipro National University, Gagarin ave., 72, Dnipro, 49010, Ukraine. Tel.: +38-097-408-25-02. E-mail: kamelina502@ukr.net*

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In the last two decades, the horse chestnut (*Aesculus hippocastanum* L.), introduced into the steppe zone of Ukraine, has been severely affected by the horse chestnut leaf miner *Camererariа ohridella* Deschka & Dimič, 1986, which results in damage to the assimilating organs, premature leaf defoliation and, as a consequence, a significant reduction in the reserve substances required for normal life of the plant. In recent studies, the main focus has been placed on the study of the pest's effects on the non-enzymatic antioxidant protection system of the representatives of the genus *Aesculus*, while the enzymatic system of horse chestnut protection from the active forms of oxygen under stress is still poorly understood. The purpose of this study was to evaluate the reaction of catalase and two peroxidases of *A. hippocastanum* leaves, which differ in the level of damage by *C. ohridella*. The intensity of damage to *A. hippocastanum* leaves by the horse chestnut leaf miner in the park zones and botanical gardens of Dnipro city was determined, the activity and isoenzyme composition of benzidine-peroxidase, activity of guaiacol-peroxidase and catalase were measured. The lowest average benzidine-peroxidase activity was found in the group of trees with low level of leaf blight and the highest activity – in the group with high level. The opposite dependence was shown by catalase, the activity of which significantly decreases with increasing level of damage inflicted by the phytophage on the chestnut's assimilating organs. Based on the determination of the variation coefficients, it has been shown that benzidine-peroxidase activity has a higher level of variability than that of catalase and guaiacol-peroxidase. It is established that under the influence of the leaf miner, activity of guaiacol-peroxidase was significantly higher by 87.1% and 75.6%, respectively, for medium and high levels of damage caused to the leaf by this phytophage as compared to that for low levels of damage. The increased level of leaf damage caused by the phytophage is reflected in the change in the isozyme profile of benzidine-peroxidase. The high activity of benzidine-peroxidase in the leaves of *A. hippocastanum* is due to the presence of several molecular forms that exhibit maximum activity in the narrow pH range (4.15–4.69). Quantitative redistribution of activity between the different molecular forms of benzidine peroxidase can be considered as the main regularity of changes in the expression of benzidine-peroxidase caused by different levels of leaf damage. The results showed that only one benzidine-peroxidase isoform with an isoelectric point of 4.15 shows a significant increase in activity (on average by 2.1 times) in *A. hippocastanum* leaves with medium and high levels of damage by *C. ohridella*. Significant reduction in activity is reported for dominant isoperoxidase with an isoelectric point of 4.25 revealing medium pest damage, and for high damage only a decreasing tendency is shown. The data obtained show that horse chestnut trees can specifically respond to mechanical damage by *C. ohridella* to leaves due to the changes in the activity of individual molecular forms of peroxidase. Further studies of oxidative metabolism are needed to understand the formation of resistance of representatives of the *Aesculus* genus to damage caused by this moth species based on a wider range of redox enzymes.

*Keywords*: benzidine-peroxidase; peroxidase isoenzymes; guajacol-peroxidase; catalase.

## Introduction

One of the unique indicators of environmental pollution is the horse chesnut (*Aesculus hippocastanum* Linnaeus, 1753), widely spread in various ecological conditions of most European cities, both in the southern and northern zones with moderate climate (Grabenweger & Grill, 2000; Steadman & Pritchard, 2004; Zerova et al., 2007). It is among the natural air filters purifying soil and water from toxic substances that are widely emitted by industrial enterprises in big cities, as well as being a valuable plant thanks to its widespread use in medicine (Apers et al., 2006; Ćalić-Dragosavac, 2010; Štajner et al., 2014). For a long time, the horse chestnut has been valued as a tree species with high-resistance to damage by insects (Gorlenko et al., 1988). But during last 20 years, the condition of chestnut plantations has severely deteriorated, as a result of the influence of adverse environmental factors (due to global climate changes and increased technogenic loading) as well as the massive reproduction of pests and phytopathogens (Kharytonov et al., 2008; Shupranova et al., 2014; Holoborodko et al., 2016; Jagiełło et al., 2017).

Horse chestnut suffers mostly from the horse chestnut leaf miner (*Cameraria ohridella* Deschka & Dimič, 1986), for which it is the main food plant. The consequence of this effect is severe damage to the leaves and premature defoliation, which adversely affects the accumulation of nutrient reserves necessary to maintain the plants' vitality in winter and restore the growth in spring (Zerova et al., 2007). The level of damage to leaves by the *C. ohridella* caterpillars in urban conditions ranges from 3.0% to 84.5% (Roginsky et al., 2014). To protect the *Aesculus* Linnaeus chestnut trees (1753) from *C. ohridella*, great expenses are allocated in Europe.

The first biological and ecological studies concerning *C. ohridella* were initiated in 1994 in Austria (Zerova et al., 2007). The female *C. ohridella* lays eggs (0.2–0.4 mm) on the upper side of the leaf along the veins (Augustin et al., 2008). Up to 100 eggs can be found on one leaf of horse chestnut (Heitland et al., 1999; Gilbert et al., 2004). The embyos develop during 2–3 weeks with 30% mortality, which depends on weather conditions (Girardoz, 2004). The caterpillar breaks the upper epidermis of the leaf, penetrates into the mesophilus and eats the cells of the palisade pa-

renchyma, reducing most of the photosynthetic active leaf tissue, bypassing the part of the blade which is rich in tannins (De Prins et al., 2003; Thalmann et al., 2003; Weryszko-Chmielewska & Haratym, 2011). The activity of photosynthesis in the necrotized leaf decreases because of the deterioration of the flows of water, minerals and organic substances.

Populations of the horse chestnut leaf miner from the Balkans and Central Europe usually produce three generations during a season (Dimič et al., 1986; Grabenweger et al., 2005). In Central Europe, the first generation appears in May, the second – in June and the third – in September. The first-generation imago is active for about a month which is synchronized with the chestnut flowering (Dimič et al., 1986). In the conditions of Dnipro city (Goloborodko et al., 2009), the development of 4 generations is registered annually (the emergence of the first imago is observed in the last decade of April, the last – from the end of October till the beginning of November). The development of a separate generation in the conditions of the city of Dnipro lasts 65–110 days. The insect only hibernates at the stage of the pupa and the pupation takes place after the first frost. Various authors have shown that the number of generations depends on the sources of nutrients and weather conditions (Baraniak et al., 2005). The studies by Stygar et al. (2017) found that the main nutrients in the leaves of horse chestnut for the caterpillars are starch and sucrose, which is confirmed by high amylase activity, as well as activity of maltase and sucrose.

Based on the analysis of 12 enzymes (esterase, amylase, malate dehydrogenase, aspartate aminotranspherase, acidphosphatase, alkaline phosphatase, malateehydrogenase, leucyl aminopeptidase, peroxidase, isocitrate dehydrogenase, alcohol dehydrogenase and octanoldehydrogenase) from 7 populations of *C. ohridella* that were collected in different places of Central Europe (Austria), it was found that the European population of the horse chestnut leaf miner is genetically homogeneous. In addition, RAPD-PCR analysis and mDNA sequencing indicate that European populations have a common ancestor (Perny, 1997).

Among the genus *Aesculus*, *A. hybrida* DC, the red horsechestnut (*A. carnea* Zeyh., 1822), red buckeye (*A. pavia* Linnaeus, 1753), yellow buckeye (*A. octandra* March.), *A. flava* Solander, 1778 (Štajner et al., 2014; Oszmianski et al., 2015), *A. indica* (Wall. excamb.) Hooker, 1859, *A. californica* (Spach) Nuttoll, *A. assamica* Griff. and *A. wilsoni* Rehder, originating in Asia and North America (D'Costa et al., 2013), are of high resistance to damage by pests. The Japanese horse chestnut (*A. turbinata* Blume) is sensitive to the chestnut moth. In some cases, mines are found on chestnut leaves that are sufficiently resistant to the chestnut (species of *A. glabra* Willd., *A. parviflora* Walt., *A. pavia* x *A. hippocastanum* and etc.). Penteluk et al. (2016) report that this fact indicates the complexity of the plant-phytophage interaction system and the fundamental impossibility of complete eradication of this pest in a big city. Additionally, due to the lack of a forage base, the caterpillar passes on to other plant species such as *Acer platanoides* Linnaeus, 1753, *A. pseudoplatanus* Linnaeus, 1753 (Mierziak, 2014), which also belong to the Sapindaceae family.

Representatives of the genus *Aesculus* are characterized by varying sensitivity to *C. ohridella*. Studies are being conducted to find out the causes of these differences (Stygar et al., 2010; Oszmianski et al., 2014; Stejner et al., 2014). Based on the analysis of three groups of substances in the leaves that can function as attractants (chloroplasts, anthocyanins), repellents (flavonoids, phenols), or as a source of food (free amino acids and carbohydrates) for several species of the genus *Aesculus* (*A. turbinata*, *Aesculus* × *neglecta* and two specimens of *A. hippocastanum*) differing in sensitivity to the leaf miner, it has been shown that high levels of anthocyanins and carbohydrates may contribute to laying eggs on leaves of sensitive *A. turbinata*. A relatively sensitive specimen of *A. hippocastanum* contained higher levels of carbohydrates and anthocyanins than a relatively stable specimen, but only during one season, which did not explain the causes of their varying sensitivity to the insect. The concentration of phenols in the sensitive *A. turbinata* and the relatively sensitive *A. hippocastanum* was higher than that in resistant *A.* × *neglecta* and relatively stable *A. hippocastanum*, respectively. This fact may indicate that it is not the total concentration of phenols, but their composition in the leaves which is responsible for the different sensitivity of *Aesculus* spp. to the horse chestnut leaf miner. The presented results show that the determined chemical compounds don't give a total picture of the biochemical relationship between *C. ohridella* and analyzed horse chestnut trees. The reaction of the enzymes-antioxidants of *A. hippocastanum* plants, whose synthesis is induced in response to an increase in the level of reactive oxygen species under stress, has not been sufficiently studied.

The aim of this study was to determine the effect of the horse chestnut leaf miner *C. ohridella* on the activity and composition of the leaf antioxidant protection enzymes of horse chestnut (*A. hippocastanum*) plants in an industrial city.

## Materials and methods

*Study area*. The research was conducted in 2018 in the city of Dnipro, Northern Ukrainian steppe (Kulbachko et al., 2015; Baranovski et al., 2016; Didur et al., 2018; Loza et al., 2018; Lykholat et al., 2018). The city is located in a zone of temperate latitudes with a rather active atmospheric circulation (the air masses predominantly moving from east to west). The climate here is temperate continental. One of the climatic features of the territory is significant fluctuations in weather conditions from year to year. Moderately wet periods alternate with sharply arid, frequent dry periods. In general, the climate is characterized by fairly cool winters and hot summers.

Eight groups of model 20–30 year old horse chestnut trees with similar morphological and taxonomic features were identified in the green plantations of the Dnipro city, as an object of research. But they had varying degrees of leaf damage by the *C. ohridella* moth (Fig. 1).

Plot 1 is located in Metallurgiv Square (48°28'26" N, 34°59'31" E), which belongs to the western Right Bank of the city and is known as having the largest accumulation of industrial enterprises.

Plot 2 is located in Manuylivs'kyy Park (48°29'13" N, 35°03'41" E, the height above sea level is 50 m) at a distance of 1.75 km to the south of the Karl Liebkneht Pipe-Rolling Plant.

Plot 3 is located in Shevchenko Park (48°27'48" N, 35°04'23" E, altitude 82.5 m). Chestnut trees are located in the central part of the park at a 100 m distance from the highway.

Plot 4 is located in the Druzhba Forest Park (48°32'02" N, 35°05'42" E, altitude 65 m) on the Left Bank of the city. The main stationary source of emissions of harmful substances into the atmosphere is the Karl Liebkneht Pipe-Rolling Plant.

Plot 5 is located in Globa Park (48°28'11" N, 35°01'48" E, altitude 56 m) in the central part of the city, which is characterized by heavy traffic.

Plot 6 is located in the territory of Molodizhnyy Park at a distance of 50 m from the highway (48°29'08" N, 34°56'42" E, altitude 82 m). It also belongs to the western Right Bank of the city and is characterized by the largest number of industrial enterprises, as well as by heavy traffic.

Plot 7 is localized in the center of the Botanical Garden of Dnipro National University (48°26'14" N, 35°02'35" E, altitude 127 m). This territory is characterized by the smallest concentration of heavy metals.

Plot 8 is located in the territory of Prydniprovs'kyy Park (48°23'59" N, 35°07'59" E, the altitude is 75 m above sea level). The largest source of emissions in this eastern part of the city is Pridniprovsk TPP, which does not show a significant impact on air pollution, as the emissions are channelled through high pipes, which leads to the removal of the zone of maximum contamination. Most of the emissions consist of toxic substances of the 3rd and 4th hazard classes, the concentration of the heavy metals is lower than that in other parts of the city (Pasichny & Serdyuk, 2002). In general, edaphotopes of the studied sites are represented by degraded ordinary chernozems having low content of humus (1.0–4.0%).

The leaves of medium formations in quantity of 5 pcs. were taken simultaneously from the lower third of southern exposure crown of five chestnut trees from each plot (an annual vegetative increment) in dry, clear weather in mid-July of 2018 (40 trees were explored in total). Infestation degree of the leaf blisters of horse chestnuts by *C. ohridella* was assessed visually during the summer period of 2018 (Zerova et al., 2007).

For biochemical analysis, the leaves were washed with water and used immediately for extraction of enzymes. Chestnut leaves (0.3 g) were homogenized in 6 ml of 0.05 M tris-HCL buffer, pH 7.4 with 0.5% polyvinylpyrrolidone (PVP), in order to isolate the enzyme preparation. Extraction was performed at +4 °C for 1 hour and centrifuged for

15 min at 14000 g. The supernatant was selected to determine the activity and isoenzyme composition of benzidine-peroxidase (BPOD) as well as the activity of guaiacol-peroxidase (GPx) and catalase (CAT). The activity of BPOD (BPOD, EC 1.11.1.7) was measured at 490 nm in the reaction mixture (0.8 mL of Na-acetic buffer, pH 5.4, 1 mL of benzidine solution and 0.2 mL of enzyme preparation) after adding 1% of $H_2O_2$. The activity was calculated in the interval of 1 min, during which the maximum reaction rate was observed (Gregory, 1966). The result was expressed in optical units (U)/g of fresh weight (FW) per minute.

BPOD isoenzyme composition was determined by the method of isoelectric focusing (IEF) in 5% horizontal polyacrylamide gel (PAAG) using the Ultrophor device (LKB, Bromma, Sweden), pH range of 3.5–6.5. The coloured gels were scanned and analyzed using the "1D Phoretix" computer program, which determined the relative content (%) for each isoform in the overall peroxidase spectrum. pH measurements were carried out directly on gels within 1 cm interval using a microelectrode (LKB 2117-111 Multiphor Surface Electrodes) at +10 °C. The values of isoelectric points (pI) of isoforms were determined from the calibration curve.


**Fig. 1.** Localization of horse chestnut plantations in the territory of Dnipro city: *1* – Metallurgiv Square; *2* – Manuylivs'kyy Park; *3* – Shevchenko Park; *4* – Druzhba Forest Park; *5* – Globa Park; *6* – Molodizhnyy Park; *7* – Botanical Garden of Dnipro National University (DNU); *8* – Prydniprovs'kyy Park

Guaiacol-dependent peroxidase activity (GPOD, EC 1.11.1.7) was evaluated according to Ranieri et al. (2001) by determining the Guaiacol oxide at 470 nm in the reaction mixture containing acetic buffer (pH 6.0), 2 mM Guaiacol solution, 0.2 mL of enzyme preparation and 0.15% $H_2O_2$. The results were calculated taking into account the molar extinction coefficient (26.6 $mM^{-1}$ $cm^{-1}$) and expressed in mM Guaiacol/g of fresh weight (FW). Catalase activity (CAT, EC 1.11.1.6) was evaluated according to Goth (1991) by measuring the optical density at 410 nm in a reaction mixture containing 0.2 mL of enzyme preparation, 0.1% of $H_2O_2$, and 4% of ammonium molybdate. The results were calculated taking into account the extinction coefficient (22.2 $M^{-1}$ $cm^{-1}$) and expressed in μM $H_2O_2$/mg protein min. The protein content of the samples was determined by the Bradford method (1976) using Coomassi brilliant blue G 250 dye (Serva, USA) against the standard bovine serum albumin (Serva, USA).

The results of the enzyme activity studies were presented as the average of x, SD (standard deviation). The data obtained were analyzed using Statistica (version 8, StatSoft, USA). The Honestly Significant Difference criterion of Tukey was used to determine the significant difference in the group averages. The differences were found to be statistically significant at $P < 0.05$. Since chestnut trees untouched by the *C. ohridella* pests were not found in the city, trees with low (8.7%) damage to the leaf were taken as the control.

## Results

The lowest (8.7%) levels of damage to leaf blades of horse chestnut trees were found in the conditions of Manuilivs'kyy Park, the recreation areas of Prydniprovs'kyy and Molodizhnyy Parks (Table 1).

**Table 1**
Damage level of chestnut horse leaves by *C. ohridella* in Dnipro city plantations

| Leaf selection Area | Manuilivs'kyy, Molodizhnyy, Prydniprovs'kyy Parks | Shevchenko Park | Globa Park, Botanical Garden | Druzhba Forest Park, Metallurgiv Square |
|---|---|---|---|---|
| Visualization |  | | | |
| Degree of leaves damage, % | 8.7 | 40.0 | 52.5 | 86.5 |

In the above conditions the decorativeness of the trees was almost never lost. 40% damaged to leaves of chestnut trees were observed in the territory of Shevchenko Park. The average level of leaves damaged by the horse chestnut leaf miner was found in Globa Park and DNU Botanical Garden. In Druzba and Metallurgiv Parks, the degree of leaf lesion averaged 86.5%, which means a significant loss of decorativeness of horse chestnut plants. Thus, in the *A. hippocastanum* plantations, there are differences in individual plant resistance, which contributes to the intraspecific differences in their metabolism under the influence of *C. ohridella.* The activity and composition of antioxidant enzymes, namely: benzidine-peroxidase (BPOD), guaiacol-peroxidase (GPOD), and catalase (CAT) were determined to find out the effects of the horse chestnut leaf miner's

influence on the functional state of the chestnut trees with various degrees of damage. The results of variance analysis of BPOD, GPOD and CAT activity of *A. hippocastanum* leaves depending on the degree of damage to the trees by pests revealed significant differences at $P < 0.05$ (Table 2).

For leaves with average damage level (49.3%) BPOD, activity was significantly higher (by 41.4%) than that of the control specimen ($F = 10.22$; $P = 2.4 \cdot 10^{-3}$; $F_{0.05} = 4.03$), and for leaves with the damage level of 86.5% activity increased by 50.0% ($F = 44.94$; $P = 3.5 \cdot 10^{-8}$). Between the medium and high leaf damage significant difference in BPOD activity was not revealed ($F = 0.292$; $P = 0.592$). The activity of guaiacol-peroxidase in chestnut leaves also increased compared to that of the control specimen, but it grew to a greater extent than for benzidine-peroxidase, namely: by 87.1 ($F = 178.86$; $P = 5.0 \cdot 10^{-28}$) and 75.6% ($F = 238.20$; $P = 3.8 \cdot 10^{-19}$) for 49.3% and 86.5%-leaf damage, respectively. A reliable decrease (by 6.1%) of GPOD activity was registered between medium and high leaf damage ($F = 4.08$; $P = 0.049$). Catalyse revealed the opposite dependency. The decrease in the activity depending on the level of leaf damage by the morse chestnut leaf miners was as follows: by 17.4 ($F = 24.24$; $P = 1.8 \cdot 10^{-8}$) and by 26.8% ($F = 55.98$; $P = 5.8 \cdot 10^{-9}$) relatively for the average and high damage to chestnut trees' leaves. Between medium and high levels of leaf damage by the pest, the activity of catalase decreased reliably by 11.5% ($F = 4.28$; $P = 0.045$).

Data analysis indicates a wide amplitude of variability of enzyme activity as to the degree of leaf damage by the pests, especially BPOD. The greatest variability in values of benzidyne-peroxidase activity was found in chestnut leaves with medium damage by the pests. The lowest variation in coefficient values was registered for GPOD activity at high damage level, and for CAT – at low level of damage by the phytophage (Table 2). When studying the activity of guaiacol-peroxidase in the homogenates of the chestnut leaves, we found a gradual decrease in the variability of activity parameters by 16.9% to 8.4% (coefficient of variation), i.e. it changes from the lowest to the highest degree of damage to leaves by the pest. On the other hand, catalase increases in variability from the lowest to the highest degree of damage to leaves by the pest (9.5% → 18.0%).

**Table 2**

Antioxidant enzyme activities (BPOD, GPOD, CAT) and values of variation coefficient (CV) in *A. hippocastanum* leaves affected by *C. ohridella* (x ± SD)

| Level of leaves damage, % | n | BPOD, U/g FW min | CV, % | n | GPOD, мМ guaiacol/g FW min | CV, % | n | CAT, μM $H_2O_2$/mg protein min | CV, % |
|---|---|---|---|---|---|---|---|---|---|
| 8.7 | 27 | $120.1 \pm 27.5^a$ | 22.9 | 27 | $4.96 \pm 0.84^a$ | 16.9 | 24 | $1.90 \pm 0.18^a$ | 9.5 |
| 49.3 | 27 | $169.9 \pm 76.0^b$ | 44.8 | 27 | $9.28 \pm 1.03^b$ | 11.1 | 24 | $1.57 \pm 0.28^b$ | 17.8 |
| 86.5 | 18 | $180.1 \pm 32.2^b$ | 17.9 | 18 | $8.71 \pm 0.73^c$ | 8.4 | 16 | $1.39 \pm 0.25^c$ | 18.0 |

*Notes*: values in column marked with different letters ($^{a, b, c}$) were significantly different according to Tukey-test $P < 0.05$; BPOD – benzidine-peroxidase; GPOD – guaiacol-peroxidase; CAT – catalase; FW – fresh weight.

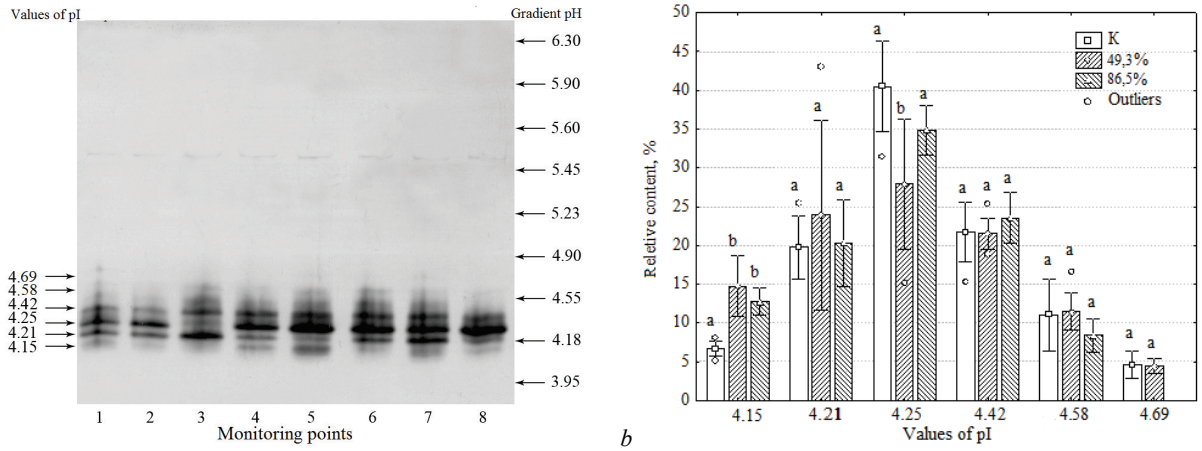


**Fig. 3.** Changes in the polyacrylamide gel isoelectric focusing (IEF) profiles of benzidine-peroxidase from chestnut horse leaves affected by *C. ohridella*: *a* – isoenzyme spectrum of benzidine-peroxidase in *A. hippocastanum* leaves; native proteins were extracted in Tris-HCl buffer, pH 7.4 containing 0.5% PVP; crude protein extracts from 0.3 g of leaf tissue were separated on a 5% IEF gel (pH 3.5–6.5); equal amounts of total protein (~3.0 μg) were loaded in each lane; the IEF gel is a representative of the analysis of samples from n = 5 trees per each monitoring point; isoelectric points of isoperoxidases are indicated on the left and pH gradient of polyacrylamide gel (PAAG) – on the right; lanes 2, 6 and 8 (Manuilivs'kyy, Molodizhnyy, Prydniprovs'kyy Parks respectively) show a low level of damage (K – control); lane 3 ( Shevchenko Park) shows 40% leaf damage; lanes 5 and 7 ( Globa Park, Botanical Garden respectively) show the average level of damage and lanes 1 and 4 (Metallurgiv Square, Druzhba Forest Park respectively) are samples with a high degree of leaf damage (average 86.5%); *b* – the relative content of BPOD isoforms (% from summary quantity isoforms of IEF spectrum) dependent on the level damage to horse chestnut leaves by *C. ohridella*; different letters (*a*, *b*) were significantly different according to Tukey-test $P < 0.05$; values represent means of n = 3 IEF spectrum

The isoenzyme composition of BPOD is characterized by a low heterogeneity (5–6 isoforms), but in different groups of trees high variability as to the relative content of individual isoforms is observed (Fig. 3).

In all plantation groups (except for Shevchenko Park), the dominant isoform was isoperoxidase with a pI of 4.25, the largest specific weight of which was observed in the groups of chestnut trees with a low level of leaf damage by the horse chestnut leaf miner and was equal to 40.5 ± 5.78% on average. In the plantations with average and high leaf infestation values, this parameter ranges 30.6–37.5%. The high relative content is registered for the molecular form of BPOD 4.42, which for the low level of damage equals to 17.1–24.5%, and for the average one – 20.3–23.3% and for the high level of leaf damage – 21.0–26.1%.

In the leaves of trees from Shevchenko Park, the dominant one is isoform with pI 4.21 (38.0%). In all other samples the value of the specific weight of this component ranged from 10.4 (Globa Park) to 25.4% (Druzhba Forest Park). Leaves damaged at 86.5% were characterized by the absence of a component with pI 4.69, which in all other samples had a small relative content (3.4–6.6%).

When plant response reaction to the damage by this pest was analyzed, a significant increase in the relative content of isoform peroxidase with pI 4.15 by 121.0% and 190.4% against the control specimen, respectively for leaves with an average and high level of destruction ($F = 36.77$; $P = 4.0 \cdot 10^{-5}$; $F = 75.73$; $P = 8.8 \cdot 10^{-7}$ respectively) was specified. A reliable difference was not found between the average and high degree of damage to leaves by the pest for this isoperoxidase.

Dominant isoperoxidase 4.25 significantly decreased by 31.0% ($F = 13.65$; $P = 2.0 \cdot 10^{-3}$) for an average degree of leaf damage by this moth. The tendency to decrease in the relative content (by 14.9%) of this component was found for leaves with high damage by the pests compared to the control specimen ($F = 4.61$; $P = 0.051$).

## Discussion

Investigation of the plant-phytophage relationship at the biochemical level is important for understanding the nature of the plants' adaptation to the action of *C. ohridella*. During the parasite's stay in the host body there are complex physiological processes between the parasite and the plant that result in the survival or death of the pest. In the case of survival, the pest affects the host organism, causing changes in metabolism, resulting in a change in the physiological state of the plant (Mithöfer & Boland, 2012). The effects of environmental stressors, including phytopathogens and pests, are accompanied by increased level of reactive oxygen species (ROS) in various leaf cell compartments (Lushchak, 2015). In response to damage to the plant caused by phytopathogens and pests, protective reactions are switched on such as barrier enhancement (lignification), gene expression, and synthesis of PR proteins, including antioxidant enzymes (Bagnoli et al., 1998). A study of the activity of antioxidant enzymes revealed sufficiently high peroxidase activity in *A. hippocastanum* leaves, which is consistent with literature data (Štajner et al., 2014). BPOD and GPOD are known to be very labile enzymes that respond to disruption of cellular homeostasis by stressors of various origins (Tognolli et al., 2002; Filho, 2006). Our studies have shown that the leaves of *A. hippocastanum* trees activate the peroxidase system to detoxify the effects of oxidative stress caused by the effects of the horse chestnut leaf miner moth. It is shown that each group of chestnut plantations, on average, is characterized by a specific type of response-reaction to leaf miner invasion. Thus, in plantations with average and high levels of damage to chestnut leaves, an increase in BPOD activity and, in particular, guaiacol peroxidase activity and a decrease in catalase activity were found, but to varying degrees.

The results obtained suggest that peroxidase activation compensates for the decrease in catalase activity. Thus, inhibition of catalase (Chen et al., 1993) is considered to be one of the main causes of hydrogen peroxide accumulation in plant tissues due to stressors. Reduced catalase is not the only possible reason for increasing the content of hydrogen peroxide in plant tissues. This effect, in particular, may be due to the increased activity of superoxide dismutase (SOD). This enzyme, which catalyzes the conversion of the superoxide radical into hydrogen peroxide, can contribute to the accumulation of the latter, especially against the background of catalase inhibition (Kolupayev et al., 2010).

The significant increase (by 87.1%) in guaiacol peroxidase activity may be due to the fact that it takes part in the processes of lignification, suberinization, auxin catabolism, wound healing, and protection against pathogenic infection (Taiz & Zeiger, 1991) which is associated with systemic plant resistance (Hammerschmidt et al., 1982; Golubenko et al., 2007). Activation of antioxidant enzymes in response to insect action is thought to be one of the key processes in the formation and development of protective responses in plant cells (Dowd et al., 1999). However, according to Filho (2006), the induction of peroxidase and polyphenoloxidase enzyme activity in response to insect attack may not be a concrete indication of the involvement of these antioxidants directly in the defense mechanisms. It has been suggested that peroxidase may participate in the regulation of the level and activity of endogenous and exogenous signaling molecules in the plant through mechanisms of synthesis and degradation of some phytohormones, peroxide compounds, and phenolic compounds (Low & Merida, 1996).

The first report on the antioxidant activity of *A. hippocastanum* compared with the resistant *A. flava* species both *in vivo* (leaves, seed embryos) and *in vitro* (androgenic embryos) was published in Štajner et al. (2014). Studies have shown that both species exhibit high antioxidant activity (based on the study of the enzymatic activity of SOD, CAT and GPOD, the amount of glutathione (GSH), flavonoids), but resistant to *C. ohridella*. *A. flava* showed higher antioxidant activity compared to the leaf of *A. hippocastanum*. However, in other species of plants, in particular, the genus *Coffee* Linnaeus (1753), the leaves of which are also severely damaged by the leaf miner *Leucoptera coffeella* (Guérin-Méneville, 1842), no association was found between peroxidase activity and the degree of damage to their parasite (Filho, 2006). Peroxidases exist as isoenzymes in different individuals of the same plant species. Each isoenzyme has variable amino acid sequences and exhibits different expression profiles, indicating their involvement in different physiological processes (Hiraga et al., 2001). According to our data, the high activity of benzidine-peroxidase in chestnut leaves is due to the presence of several molecular forms that exhibit maximum activity in the narrow pH range (4.15–4.69). Overall, despite the strong effect of the horse chestnut leaf miner on leaves, the structure of the IEF spectrum of BPOD in all the tested chestnut leaf samples was stable but differed significantly in the expression/specificity profiles of peroxidase isoenzymes. The latter testifies to the unequal role of individual peroxidase isoforms in the process of adaptation of chestnut plants to *C. ohridella*.

It should be noted that the unequal activity of peroxidase was characteristic of tree groups with the same level of leaf damage by the phytophage. Thus, in the group of plants with low leaf damage, two plantations (Manuilivs'kyy and Prydniprovs'kyy Parks) had the highest relative isoperoxidase content with a pI of 4.25 (41.6% and 46.3%, respectively), and in the trees from the monitoring plot in Molodizhnyy Park (33.7%). The high expressiveness of isoform 4.25 was noted for trees from Globa Park, the Botanical Gardens (average leaf damage) and Druzhba Forest Park (high leaf damage). There was a significant increase in the activity of isoperoxidase from PI 4.15, the relative content of which in leaves with average and high damage was higher by 2.2 and 1.9 times, respectively, compared to that of the leaves with low level of damage. It can be assumed that this isoform of peroxidase may be involved in the processes of interaction of the plant cell with the pest. According to the literature, the effect of insects and pathogenic infection on the quantitative and qualitative composition of isoenzymes of antioxidant enzymes has been identified (Allison & Schultz, 2004).

Thus, by changing the expressiveness/relative content of the isoenzyme peroxidase system of chestnut plants, the trees attempt to ensure the resistance to the action of phytophages and to regulate the homeostasis. However, the enzymatic antioxidant system of protection has proved to be ineffective in most plantations, despite the increased activity of BPOD and GPOD, possibly due to excess accumulation of reactive oxygen forms as well as hydrogen peroxide, as indicated by a significant decrease in catalase activity.

The differences in the expressiveness/specific gravity of horse chestnut peroxidase isoforms which are affected by the horse chestnut leaf miner can be explained by the different conditions of their existence in a large industrial city, which include the effects of both emissions of road transport (Globy Park, Druzhby Forest Park) and industrial enterprises (Metallurgiv Square, Shevchenko Park). For example, highways with heavy traffic pass near Metallurgiv Square. The highest concentrations of heavy metals in soils and air pollution by gas and dust emissions are observed in the area. Shevchenko Park is in the zone of intensive sedimentation of the impurities of the factories of the southeastern part of the Livoberezhzhya (Pasichnyy & Serdyuk, 2002), and Globa Park belongs to the central part of the city, which is characterized by intensive traffic of vehicles.

## Conclusion

The results obtained demonstrate the activation of the enzymatic antioxidant protection system of the horse chestnut to the damaging effect of the horse chestnut leaf miner, which allows the plant to survive and complete the programme of ontogeny in adverse conditions. The most significant protection of cells from this moth is provided by increase in the activity of guaiacol-peroxidase, which indicates an increase in the barrier properties of cells. In order to fully understand the process of protecting the horse chestnut from the pest in the future, special attention should be paid to the study of the activity of superoxide dismutase, as the primary link to neutralize the active forms of oxygen, the content and composition of leaf proteins in order to identify macromolecules with protective properties. The influence of the urbogenic environment (emissions from road transport, industrial enterprises) on the metabolism of the antioxidant enzymes of the assimilating organs of *A. hippocastanum* trees is not excluded, which requires further studies of the combined action of the abiotic and biotic environmental factors which are adverse for horse chestnuts.

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