

Effect of *Cameraria ohridella* on accumulation of proteins, peroxidase activity and composition in *Aesculus hippocastanum* leaves

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This study analyzed the dynamics of leaf damage, the content of easily soluble proteins, benzidine-peroxidase activity and the composition of acid enzyme isoforms in the leaves of *Aesculus hippocastanum* L. under the influence of the chestnut miner *Cameraria ohridella* Deschka et Dimić (Lepidoptera, Gracillariidae) in plantations in one of the Ukraine's largest industrial cities Dnipro, located in the steppe zone of Ukraine. During July-August, the destruction of leaves by the pest in relatively stable horse chestnut trees increased to 13.3%, while in unstable trees it increased to 97.5%. The maximum amount of protein was found in horse chestnut leaves in July, which decreased significantly in August and correlated with the level of leaf damage by the pest. The horse chestnut leaves were characterized by a more than twofold increase in cytoplasmic peroxidase activity in cases of high level of damage to the leaves by the phytophage. These observations indicate that consumption of the leaves by *C. ohridella* causes oxidative stress, which leads to the activation of enzyme. The horse chestnut trees have been shown to respond specifically to the attack of *C. ohridella* due to changes in the activity of individual peroxidase isozymes, the expression of which varies across the spectrum. In the leaves of the horse chestnut trees with a high level of damage by miner, the expression of enzyme isoforms in the pH range of 4.08–4.15 is significantly activated, which can be considered as a reliable biochemical marker of plant sensitivity of *Ae. hippocastanum* to phytophagous attack by *C. ohridella*. However, increase in the degree of phytophage invasion does not change the activity of isoperoxidase 4.21, and isoperoxidase with pI 4.25, 4.42 and 4.58 correspond to a decrease in activity compared with relatively resistant *Ae. hippocastanum* plants. We noted that the high total activity of peroxidase, as well as active adjustments in the spectrum of the peroxidase system, obviously, contribute to maintaining the functional integrity of the photosynthetic system of leaves of *Ae. hippocastanum* under the influence of the chestnut miner *C. ohridella* by neutralizing reactive oxygen species.

Keywords: horse chestnut; insect herbivore; leaf miner; oxidative enzyme; plant defence.

Introductions

Aesculus hippocastanum L. is widely planted along streets and in parks as an ornamental tree, and it is one of the most studied plant species in relation to air pollution by aeropolutants (Petrova et al., 2012; Shupranova et al., 2014). In recent decades horse chestnuts have been severely affected by the horse chestnut leaf miner (HCLM) *Cameraria ohridella* et Dimić (Deschka & Dimić, 1986; Akymov & Zerova, 2003; Weryszko-Chmielewska & Haratym, 2011). In the conditions of Dnipro city (the Northern steppe subzone of Ukraine) development of four generations is registered annually (emerging of first-generation imago is observed in the last decade of April, the last in late October and early November). The development period of a separate generation in Dnipro city lasts 65–110 days (Holoborodko et al., 2009). At mass invasion of leaves numerous mines occur on them that greatly impair the horse chestnut tree's appearance and weaken the trees. The origin of this phytophage remained unclear for a long time, since it was not found in places where the *Aesculus* genus naturally grows, either in East Asia or in America. Its origin from the Southern Balkans (Albania, Macedonia, and Greece) has been proven based on genetic analysis (Valade et al., 2009), which showed that the genetic diversity of the species is greatest in the places where it was firstly discovered. From there, for an unknown reason, one of the species' haplotypes began to spread and formed current wide range.

This insect pest was listed among the 100 worst invasive species of Europe (Augustin, 2009), and its biology, ecology, physiology, biochemi-

stry and effective management are actively studied (Baraniak et al., 2005; Barta, 2018; Holoborodko et al., 2009, 2016; Stygar et al., 2010; Rämert et al., 2011; Shupranova et al., 2019). There are known attempts to control the spread of the miner (Kuldová et al., 2007; Sukovata et al., 2011), which unfortunately were not effective enough. Therefore, an alternative approach to the control of miner reproduction using fungal pathogens (entomopathogenic fungi) has been considered in recent years (Schemmer et al., 2016; Barta, 2018). In the study of the horse chestnut plant's reaction to the effects of phytophage, the importance of the chemical component of plant defense systems is emphasized, which includes secondary metabolites (alkaloids, terpenoids, cyanogenic glycosides, flavonoids, tannins, oxycoric acids), antioxidant defense enzymes involved in blocking of signal transmission processes through the plant, inhibiting metabolism, increasing the resource and energy input for nutrient absorption (Mithöfer & Boland, 2012; Hryhoryuk & Luk'yanenko, 2015), neutralization of reactive oxygen species (Stajner et al., 2014; Shupranova et al., 2019).

Studying the repeated effect of HCLM defoliation on horse chestnut reproduction, Thalmann et al. (2003) concluded a strong decrease in the storing of the assimilates by seeds in infected trees versus control. Mathematical calculations of models on HCLM-infected trees in Northern Italy showed that photosynthetic net losses of primary production of infected trees were about 30% annually (Nardini et al., 2004). Such processes can seriously impair the future growth and survival of horse chestnut seedlings. Like all animals, herbivorous insects require a wide range of nutrients. Research of Stygar et al. (2010) found that the main nutrients for

the caterpillar in horse chestnut leaves were starch and sucrose, which is confirmed by high amylase activity, as well as maltase and sucrose activity. The second important class of macromolecules are soluble proteins that can influence insect productivity along with carbohydrates, including growth and reproduction rates (Behmer & Joern, 2008; Behmer, 2009; Roeder & Behmer, 2014), tolerance to plant toxins (Patel et al., 2013; Deans et al., 2016), and pathogens (Lee et al., 2006; Povey et al., 2009).

Despite the ecological significance of plant soluble proteins in the formation of plant-insect interactions, the study of their content in the horse chestnut is insufficient. For these reasons, documenting the concentration and variability of highly soluble proteins and antioxidant enzymes is important for understanding both the food ecology of herbivorous insects and for protecting plants against parasitic insect attack.

The goal of the work was to evaluate the influence of mining moths on the content of highly soluble proteins, activity and composition of benzidine-peroxidase in the leaves of *Ae. hippocastanum* L. trees.

Materials and methods

Horse chestnut leaves were collected in mid-July and August 2018 from eight plantings of different purposes in Dnipro city (48°27'00" N, 34°58'59" E, Northern subzone of the steppe of Ukraine), the location of which is shown in Figure 1, and their characteristics are given in Table 1. Measurements were made during mass infection by the third-generation *C. ohridella*. In the conditions of the Dnepropetrovsk Oblast *Ae. hippocastanum* belongs to a group of drought-resistant, winter-hardy woody plants with isolated cases of freezing of annual shoots.

Five trees were analyzed on each site. Leaves (5–7 pieces) were selected from the same location of the tree crown. The degree of leaf damage was assessed visually by Zerova et al. (2007). Only green parts of the leaf with no mines were taken for analysis. After visual evaluation, the leaves were used for biochemical studies. Plant material (0.3 g) was extracted with 6 mL 0.1 M Tris HCl buffer (pH 7.4) at +4 °C.

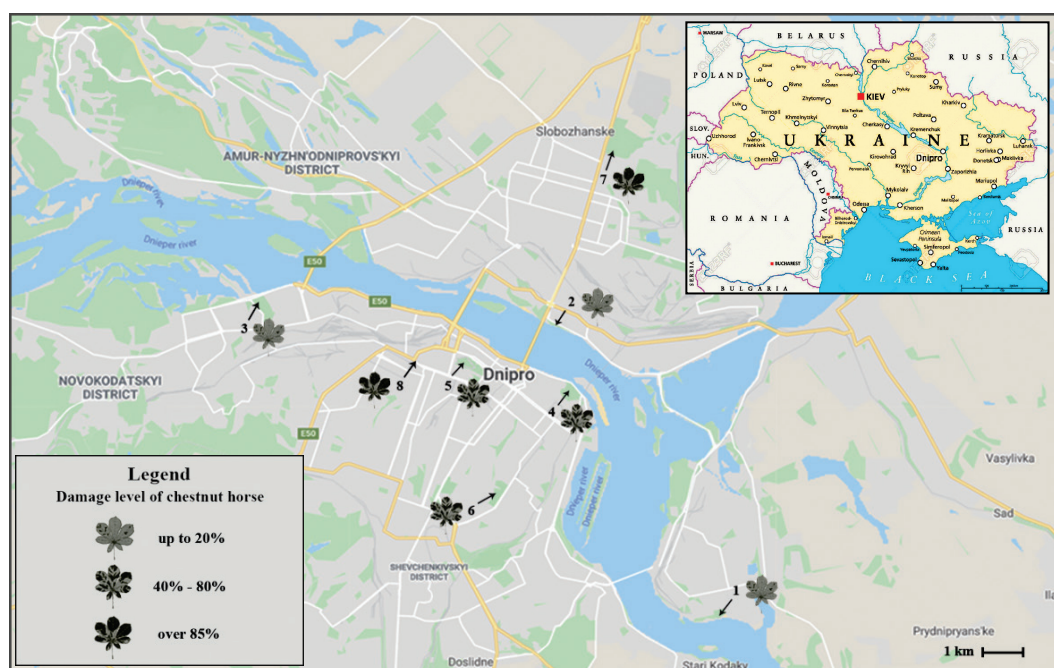


Fig. 1. Locations of sampling sites of the horse chestnut in Dnipro city

Table 1
Characteristics of trial areas

Plot number	Location	Park coordinates	Altitude above sea level, m	Visualization	Plantation groups by leaf damage levels
1	Prydniprovskyy Park	48°23'59" N, 35°07'59" E	75		I
2	Manuylivskyy Park	48°29'13" N, 35°03'41" E	50		July 8.7% August 13.3%
3	Molodizhnyy Park	48°29'08" N, 34°56'42" E	82		
4	Shevchenko Park	48°27'48" N, 35°04'23" E	82.5		II
5	Globa Park	48°28'11" N, 35°01'48" E	56		July 49.3% August 78.3%
6	Botanical Garden of DNU	48°26'14" N, 35°02'35" E	127		
7	Druzhba Forest Park	48°32'02" N, 35°05'42" E	65		III
8	Metallurgiv Square	48°28'26" N, 34°59'31" E	65		July 86.5% August 97.5%

Extracts were centrifuged at 14000 g 20 min, and were used to determine content of highly soluble proteins (ESP) and activity and composition of benzidine-peroxidase. The activity of benzidine-peroxidase (BPOD, EC 1.11.1.7) was determined by Gregory (1966). Changes in op-

tical density were registered for 1 min at 490 nm after adding 1% H₂O₂ to the reaction mixture (acetic buffer, pH 6.0, 0.02 mm benzidine, and 0.2 mL of the sample). The result was expressed in absorbance units/g of raw material (RM). Protein content was determined with colorimetric method for total coarse protein extract of the sample with Bradford (1976) method using bovine serum albumin as standard; the result was expressed in mg/g of the sample weight. Isoenzyme composition of peroxidase was determined by isoelectric focusing (IEF) in a 5% horizontal polyacrylamide gel (PAAG) using Ultraphor (LKB, Bromma, Sweden), pH range 3.5–6.5. Measurements of pH gradient were performed directly on the gel with a 1 cm interval using a microelectrode (LKB 2117-111 Multiphor Surface Electrodes) at +10 °C. The values of isoelectric points (pI) of the isoforms were determined using calibration curve. The coloured gels were scanned and analyzed using the 1D Phoretix software.

Results were presented as average for each biochemical procedure. Data obtained were analyzed using the Statistica (version 8, StatSoft, USA). Tukey's Honestly Significant Difference test was used to determine the significant difference between the group means. The differences were found to be statistically significant at P < 0.05.

Results

The experiments were conducted in seven park areas and Oles Honchar Dnipro National University Botanical Garden; they were pooled into three groups (I–III) according to the level of damage by the horse chestnut miner *C. ohridella* (Fig. 2).

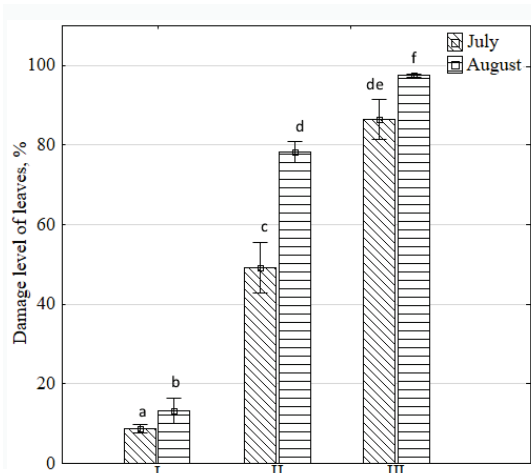


Fig. 2. Changes in the damage level of *Ae. hippocastanum* leaves affected by the horse chestnut miner *C. ohridella* in 2018 ($x \pm SD$, $n = 9$): group I – 8.67 and 13.3%; group II – 49.3% and 78.3%; group III – 86.5% and 97.5% respectively in July and August

Environmental conditions in 2018 were favourable for the development of horse chestnut miner, so leaf damage by the pest in plantings in July was 8.7–86.5%, and in August 13.3–97.5%. Group I includes relatively stable (conditional control) horse chestnut trees damaged in July by 8.7% and in August by 13.3%, and groups II and III include the trees that were respectively sensitive and highly sensitive to *C. ohridella*.

The level of soluble leaf proteins in July was almost the same in all the studied groups of plantings and was 2.74 mg/g (group I and II of plantings) and 2.71 mg/g (group III of plantings). Later in August we observed a decrease in protein content correlated with an increase in the level of leaf destruction by horse chestnut miner (Fig. 3).

Compared to July, in the conditional control plantings (group I of plantings) this decrease was 15.0% ($F = 56.67$; $P = 2.9 \cdot 10^{-10}$), in the II group of plantings a significant decrease was already 28.8% ($F = 73.1$; $P = 6.5 \cdot 10^{-12}$) and in the III group it was 43.9% ($F = 156.2$; $P = 5.0 \cdot 10^{-15}$). In August, a decrease in protein level in the II group of plantings compared to the conditional control was 16.3% ($F = 29.4$; $P = 2.6 \cdot 10^{-5}$), and in the III group it was 34.8% ($F = 170.2$; $P = 1.3 \cdot 10^{-18}$). There was also a difference in the protein content in the leaves of horse chestnut trees between groups II and III of plantings, namely: a decrease of 22.1% ($F = 16.2$; $P = 1.8 \cdot 10^{-4}$).

Results of a dispersion analysis of BPOD activity dependence on the degree of damage of horse chestnut leaves by the pest in August revealed significant differences at $P < 0.05$ (Table 2).

Table 2

Peroxidase activity and values of variation coefficient in *Ae. hippocastanum* leaves affected by *C. ohridella* ($x \pm SD$)

Level of leaves damage, % (August)	n	BPOD, U/g FW min	CV, %
13.3	27	194.1 ± 32.2 ^a	16.6
78.3	27	423.4 ± 68.8 ^b	16.3
97.5	18	414.4 ± 67.1 ^b	16.2

Notes: values in column marked with different letters ^a and ^b were significantly different according to Tukey-test $P < 0.05$; BPOD – benzidine-peroxidase; FW – fresh weight.

Depending on the level of leaf damage by the caterpillar compared to the conditional control, the activity of peroxidase was 2.2 times more ($F = 109.71$; $P = 1.4 \cdot 10^{-8}$) in group II (78.3% of damage) and 2.1 times more ($F = 115.14$; $P = 7.9 \cdot 10^{-8}$) in group III of horse chestnut plantations (97.5% of leaf damage). No significant difference in enzyme activity was found between groups II and III of plantings ($F = 0.10$; $P = 0.762$). Variability of this parameter for all plantings was stable and amounted to 16.2–16.6%.

In the spectrum of cytoplasmic peroxidase of horse chestnut leaves in August, 5 isoenzymes were detected within the pH range 4.08–4.69 (Fig. 4). Isoperoxidase with a pI of 4.25 was dominant in the leaves of all the trees studied (except planting 2).

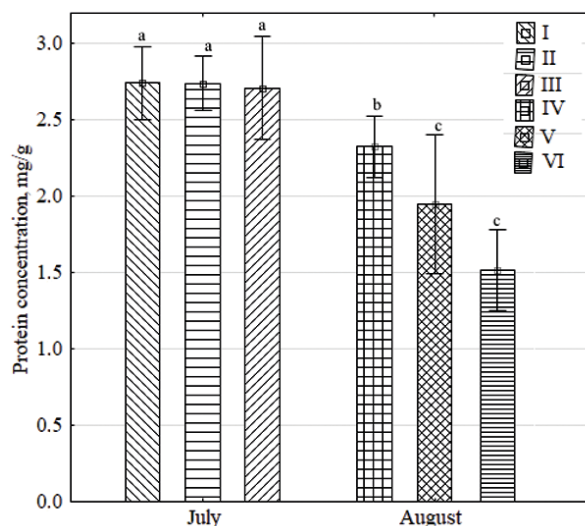


Fig. 3. Differences in the soluble protein levels of chestnut horse leaves affected by *C. ohridella*: native proteins were extracted from 0.3 g leaves tissue in 6 mL of Tris-HCl buffer, pH 7.4 containing 0.5% PVP; protein content is a representative of the analysis of samples from $n = 5$ trees per each monitoring point; July – lane I shows a low level of damage (8.7%); lane II shows an average level of damage (49.3%); lane III shows a high level of damage (86.5%); August – lane IV are samples with a low degree of leaf damage (13.3%); lane V and VI show a high level of damage (78.3% and 97.5% respectively); different letters ^a, ^b, ^c were significantly different according to Tukey-test $P < 0.05$; values represent means of $n = 27$ (lanes I, II, IV and V) and $n = 18$ (lanes III and VI)

Analysis of the IEF spectrum of peroxidase in the soluble protein fraction showed differences in their relative content in *Ae. hippocastanum* leaves with various degrees of damage caused by horse chestnut miner (Table 3).

Table 3

Relative content of BPOD isoforms in *Ae. hippocastanum* leaves affected by *C. ohridella* ($n = 3$; $x \pm SD$)

Level of leaves damage, %	pI values of isoperoxidases				
	4.08–4.15	4.21	4.25	4.42	4.58
13.3	11.5 ± 1.91 ^a	19.8 ± 1.13 ^a	36.8 ± 1.66 ^a	28.3 ± 3.95 ^a	5.36 ± 1.04 ^a
78.3	19.2 ± 5.25 ^{ab}	19.8 ± 9.53 ^a	28.9 ± 9.64 ^a	24.5 ± 6.07 ^a	3.74 ± 1.12 ^{ab}
97.5	24.9 ± 0.43 ^b	17.0 ± 3.40 ^a	29.9 ± 1.64 ^a	26.0 ± 0.50 ^a	2.99 ± 0.13 ^b

Notes: values in column marked with different letters ^a, ^b were significantly different according to Tukey-test $P < 0.05$.

With increasing levels of leaf damage by the pest a pronounced increase in the activity of isoforms occurs within the pH range 4.08 to 4.15 (Fig. 4b) compared with conventional control (group I of plantings) on 67.0% for group II of plantings (the difference is statistically insignificant) and on 119.5% for group III plantings ($F = 58.43$; $P = 1.6 \cdot 10^{-3}$). A significant decrease (by 48.9%) in the specific weight was registered for the isoform with pI 4.58 in the III group of plantings compared to the conditional control ($F = 15.5$; $P = 0.017$). Expression of the peroxidase isoform with pI 4.21 was almost identical in groups I and II and decreased by 16% in group III of plantings. The main isoform with pI 4.25 showed a tendency to decrease in trees with high leaf damage by phytophage (groups II and III) compared to low (group I), namely by 21.5 and 18.7%, respectively. The same trend was revealed in isoform with pI 4.42: a decrease by 13.4 and 8.1% in comparison with the conditional control in accordance with the II and III groups of plantings.

Discussion

Plants are exposed to many types of mechanical stresses caused by both biotic and abiotic factors. Biotic causes of mechanical injuries include insect attacks, infestations by parasites and many organisms that feed on plants that have evolved mechanisms to protect themselves from mecha-

nical injuries during their evolution (Ashry & Mohamed, 2011; Mohamed et al., 2012; War et al., 2018). Peroxidases and easily soluble proteins are

among the factors that actively respond to cell damage by insects (Esteban-Carrasco et al., 2001; Deans et al., 2016).

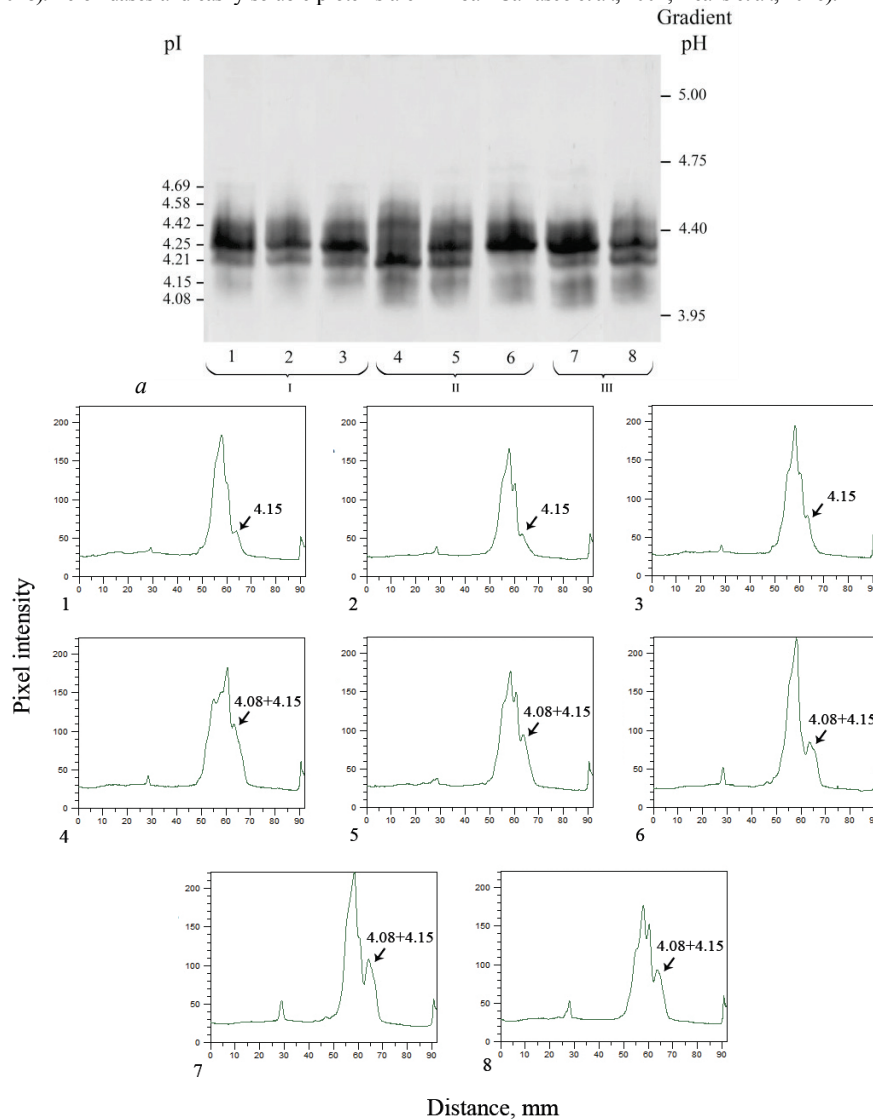


Fig. 4. Changes in the polyacrylamide gel isoelectric focusing (IEF) profiles of benzidine-peroxidase from chestnut horse leaves affected by *C. ohridella* in August. *a* – isoenzyme spectrum of benzidine-peroxidase in *Ae. hippocastanum* leaves; native proteins were extracted in Tris-HCl buffer, pH 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; lanes 1, 2 and 3 show a low level of damage (13.3%); lanes 4, 5 and 6 with 78.3% damage leaves; lanes 7 and 8 are samples with a high degree (97.5%) of leaf damage;

b – densitograms of IEF peroxidase profiles from horse chestnut leaves (designations 1–8 as in Fig. 1); arrows indicate notable differences in levels of isoform peroxidase intensity with pI 4.08–4.15

This study identified significant differences in the content of soluble proteins and the activity and composition of peroxidase according to leaf damage degree of horse chestnuts caused by *C. ohridella*. Our research showed a decrease in the content of easily soluble proteins of *Ae. hippocastanum* leaves in August. It can be assumed that the protein content is significantly reduced in leaves infected by insects, because the plant reduces the rate of protein synthesis under biotic stress, and the entire translation mechanism is shifted to the production of proteins associated with protection (Le Gall, 2014). This may be the reason why the protein content in the leaves decreases, and the activity of peroxidase increases. In addition, a decrease in protein levels in August may be associated with the outflow of biomolecules toward the storage organs of the plant. Reducing the content of soluble proteins in the phase of active outflow of assimilates is a normal physiological process, but in a leaf damaged by a pest this process can be accelerated compared to control. The induction of antioxidant enzymes in plants through the attack of herbivorous insects has received considerable attention in recent years (Gill et al., 2010; Gulsen et al., 2010). Peroxidases are induced in many plants in response to insect attack and are an important component of the direct response to plant

damage, as they regulate a number of processes directly or indirectly related to plant resistance to insect pests (War et al., 2018). Our previous study (Shupranova et al., 2019) carried out in July 2018 found an increase in the activity of benzidine peroxidase due to HCLM effect by an average of 46.0%. Our results showed that the activity of peroxidase in the leaves of horse chestnut plants in August from the same experimental sites but with a higher level of damage (78.3% and 97.5%) is significantly higher (2.2 and 2.1 times, respectively) than in the leaves of relatively resistant trees with damage level 13.3%. The increase in cytoplasmic peroxidase activity caused by mechanical injury was within the range of the increase in peroxidase activity found in other plants (Singh et al., 2013; Sánchez-Sánchez & Morquecho-Contreras, 2017). The changes observed in the activity of the enzyme in phase of active outflow of assimilates indicates its active participation in metabolic processes of leaves after damage caused by phytophages.

A wide range of peroxidase responses to various response producers is known to be associated with a large number of peroxidase isoforms and their multiple functions in the cell (Campa, 1991; Otter & Polle, 1997; Nagy et al., 2004; Lykholat et al., 2018). Acidic and basic isoperoxidases

are involved in the stress state of the plant organism. In one scientific work (Andreeva, 1988) it is shown that the main peroxidases are activated at the beginning of infection, and changes associated with the metabolism of auxin and ethylene induce increased synthesis of acidic isoenzymes as a later stage of response or defense. We have shown that relative content of isoforms in peroxidase across the entire spectrum changes in leaves after *C. ohridella* impact. In the leaves of horse chestnut trees with a high level of damage by miners, expression of BPOD isoforms is activated in the pH range of 4.08–4.15. So, in August, and also as it was found in the work (Shupranova et al., 2019), the expression of the most acidic isoperoxidases significantly increases in plants unresistant to *C. ohridella*. At the same time, an increase in the degree of phytophage invasion practically does not change the activity of isoperoxidase 4.21, while the molecular forms of the enzyme with pI 4.25, 4.42 and 4.58 respond with a decrease in activity compared to plants relatively resistant to *Ae. hippocastanum*.

Our results suggest that degradation of soluble proteins and increased peroxidase activity in horse chestnut leaves may be caused by oxidative stress. Activation of BPOD is aimed at H₂O₂ destruction and counteracting oxidative stress caused by *C. ohridella* attack. It is shown that changes in physiological processes of plant leaves under adverse conditions, particularly photosynthesis correlates with peroxidase activity (Kim et al., 2010; Smolinska, 2017). This enzyme destroys ROS and protects chloroplasts from damage. Therefore, it is suggested that the strategy of survival of horse chestnut from *C. ohridella* attack consists in maintaining the process of photosynthesis in the areas of leaves without mines to provide the next offspring with spare substances. Our assumption is supported by the results of Raimondo et al. (2003), in which it was found that chloroplasts inside the green areas of the leaf mined by *C. ohridella* larvae remained intact, and the intensity of photosynthesis in these areas was close to the unmined leaves.

Conclusions

Leaf miner *C. ohridella* affects the functional state of horse chestnut confirmed by changes in the content of soluble proteins, activity and isoenzyme composition of benzidine peroxidase during the growing season. The study of dynamics of easily soluble proteins showed their decrease because of a high level of *Ae. hippocastanum* leaf damage. Statistically significant differences were found for soluble peroxidase whose activity increased by an average of 2.1 times due to the high pest damage of leaves of horse chestnut trees. High level of leaf damage by the phytophage was reflected in the change in the isozymic profile of benzidine peroxidase. The main regularity in the negative influence of *C. ohridella* is a significant increase in activity of the most acidic molecular forms (pI 4.08–4.15) of cytoplasmic peroxidase in horse chestnut leaves. Understanding the patterns of the molecular nature of *Ae. hippocastanum* adaptation to phytophage effect is a prerequisite for development effective forms of this species resistant to *C. ohridella*.

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