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Antioxidant activity and resistance traits of *Aesculus* species against *Cameraria ohridella* Deschka & Dimić infestation in the Ukraine steppe

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Abstract: The study aimed to assess the enzymatic antioxidant system for determining the species-specific response of *Aesculus* species different in tolerance to *C. ohridella* attacks. Antioxidant activity of non-infested and infested leaves in six species of the genus *Aesculus* was investigated on 15-year-old representatives growing in Dnipro city (Steppe zone, Ukraine). The leaves of *Aesculus* both healthy and mined by *C. ohridella* were sampled within Dnipro city in 2023. Activity of benzidine and guaiacol peroxidase (BPx, GPx), catalase (CAT), and content of soluble proteins (SP) were determined. The isoenzyme composition of BPx was determined by isoelectric focusing (IEF). Statistical differences between the species were corroborated via ANOVA using Statistica software. High constitutive levels of BPx, GPx activities and soluble protein concentrations were detected in the leaves of *Aesculus parviflora*; this species was highly resistant to the leaf miner infestation compared to unresistant *Ae. hippocastanum* and moderately resistant species. Different adaptative ways of *Aesculus* species to *C. ohridella* influence were shown to occur due to the diverse functional role of oxidative enzymes. In *A. glabra*, catalase was activated; induction of this enzyme occurs only at high ROS concentrations. BPX stimulation found in *A. hippocastanum* and *A. flava* could indicate the participation of this enzyme in hydrogen peroxide neutralization and synthesis of phenolic compounds. Increased GPx activity (in *A. pavia*) occurred mainly (except for the disposal of H_2O_2) to strengthen the cell wall; it enhanced the resistance response against the influence of the horse-chestnut leaf miner. Under the influence of the oligophagous insect, only quantitative changes in IEF profiles of isoperoxidase were recorded specifically depended on the species of *Aesculus*. This output may be beneficial for application of *Aesculus* plants (*A. parviflora, A. × carnea, A. flava*) highly resistant to stress in urban green plantings. This study showed that the antioxidant status of leaves in the genus *Aesculus* can be a reliable indicator of the plant tolerance to *C. ohridella* attacks.

Keywords *Cameraria ohridella*, protein content, *Aesculus* genus, horse chestnut resistance, peroxidase and catalase activities, peroxidase IEF profiles

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Introduction

The horse chestnut leaf miner *Cameraria ohridella* Deschka & Dimić (Lepidoptera: Gracillariidae) is an invasive vicious pest that feeds on the white horse chestnut *Aesculus hippocastanum* L.; the insect spread rapidly from its original habitat (mountain forests of Albania, Bulgaria and Greece) and poses significant environmental and economic challenges in Europe (Freer-Smith & Webber, 2017; Haubrock et al., 2021). The most likely period of the chestnut moth-miner invasion into Ukraine is considered to be 1998 year; its expansion ocurred from Hungary (Zerova et al., 2007). In Dnipro city, the development of 4 generations was annually registered. The emerging of first-generation imagos was observed in the last decade of April, and the last generation in late October – early November. Duration of development period of a separate generation in Dnipro city was 65 to 110 days (Holoborodko et al., 2016). *C. ohridella* is characterized by the presence of a sufficient food resources, the absence of natural enemies; development of this insect causes dehydration, yellowing, drying of leaves and premature death of *A. hippocastanum* trees in natural conditions. Its larvae mine leaves and, in appropriate conditions, is able to damage up to 100% of the leaf area (Bačovský et al., 2017). Nevertheless, horse chestnut trees are still often planted both in cities and in mixed forests of Europe (Gubka et al., 2024). Feeding the miner caterpillars on the palisade parenchyma causes a reduction on most photosynthetically active leaf tissue, and thereby negatively affects accumulation of nutrient reserves due to shortage of the flows of water, organic substances and minerals (Weryszko-Chmielewska & Haratym, 2011). Recently, *Ae. hippocastanum* is considered to be a vulnerable species threatened with extinction in nature and listed in the World Red List and the European Red List of trees (Allen & Khela, 2017).

Finding and expanding the range of pest-resistant species and their optimal placement in urban landscaping is important measure for effective use in improving the environment and providing other ecosystem services to the population (Miroshnyk et al., 2022; Wang et al., 2019). According to modern views, identification of new resistant donors and the growing of resistant varieties by the way of breeding to adopt the plant to urban environment can be the safest and most cost-effective alternative to the fighting against *C. ohridella* attacks (Pati et al., 2023; Gubka et al., 2024). According to the research by Gubka et al. (2024), horse chestnut trees of the variety 'Mertelík' were not heavily infested by *C. ohridella* larvae, which leads to the conclusion that it may be suitable for use in the field conditions. In addition, individual trees were identified among various *Ae. hippocastanum* trees that significantly differed in resistance to colonization by *C. ohridella* (Irzykowska et al., 2013; Paterska et al., 2017; Materska et al., 2022).

Among the species of the genus *Aesculus*, Asian endemic horse chestnut species *A. assamica*, *A. chinensis*, *A. indica* have increased resistance to leafhoppers. North American species such as *A. californica*, *A. flava*, *A. carnea*, *A. glabra*, *A. parviflora*, *A. pavia* and *A. sylvatica* have varying degrees of resistance traits to foliar damage by chestnut moth (Ozsmiański et al., 2015; Paterska et al., 2017; Lesovoy et al., 2020). *C. ohridella* belongs to oligophagous insects; this pest is able to complete its full generation on a number of woody species, such as *A. hippocastanum*, *A. turbinata* Blume (D'Costa et al., 2014) and *A. glabra* Willdenow (Walczak et al., 2017), as well as on *Acer pseudoplatanus* (Péré et al., 2010), which belongs to the same order as *Aesculus* species (Sapindales). Péré et al. (2010) noted that *C. ohridella* females can also lay eggs on many other woody species, but mainly on trees that grow near *A. hippocastanum*, and with a frequency much lower than on *A. hippocastanum*. Kenis et al. (2003) found that most of Ohrid moth larvae die during their development on such resistant species as red buckeye *A. pavia*, yellow buckeye *A. flava*, Ohio buckeye *A. glabra*. According to the studies conducted in Ukraine during 2008–2019 (Forest-steppe zone), red horse chestnut *Aesculus × carnea* Hayne (hybrid *A. hippocastanum × A. pavia*), *A. flava* Sol., *A. pavia* and *A. parviflora* have increased resistance to *C. ohridella* attacks (Lesovoy et al., 2020). Bottlebrush buckeye *A. parviflora* is a large ornamental shrub; it is unique among other chestnut species because it keeps its foliage in good condition until autumn despite the sensitivity to sunburns, and it is more resistant to diseases and insect attacks than most of the other *Aesculus* species. *A. × carnea* is rarely considered to be a host plant for *C. ohridella* because, despite the abundant oviposition by females of each generation, larvae usually die within leaf tissues before reaching the third stage (Kukuła-Młynarczyk et al., 2006). However, Dzięgielewska et al. (2007) found that the horse chestnut leaf miner was able to develop two full generations on *A. × carnea* under some following circumstances: the presence of heavy-infested *A. hippocastanum* trees in the vicinity, high abundance of *C. ohridella* population, and mild winters in several consecutive years at the location. Nevertheless, the larvae number and foliar damage degree in *A. × carnea* remained low, and maximum damage of the leaves reached 10% throughout the vegetative period.

The stress caused by herbivorous insects disrupts a number of biochemical processes involved in the mechanisms of resistance in woody plants. Therefore, a deep understanding of the underlying mechanisms of resistance is crucial for identifying and using novel phytophagous insect-resistant plants in reforestation and green building programs (Gill et al., 2010; Zhao et al., 2016). One of such mechanisms is the induction of antioxidant defense enzymes in response to phytophagous insect attacks. So far, information on their diverse role in development of the sustainability of *Aesculus* species remains limited.

The plants damaged by pathogens and insect pests often exhibit elevated concentrations of reactive oxygen species (ROS) (Akbar et al., 2023). ROS are generated during the activation of plant immune response. They mediate the interplay between constitutive and induced defenses, which leads to the activation of phytohormones. Acting in high concentrations, they destroy the structures of proteins, DNA, resulting in chlorophyll degradation and leaf necrosis. The degradation of reactive oxygen species occurs via two systems, enzymatic and nonenzymatic. The enzymatic process in plants occurs through a number of antioxidant enzymes involved in multiple biochemical reactions, among which catalase (CAT) and peroxidase (BPx) are especially important. Peroxidases are heme-containing glycoproteins that neutralize hydrogen peroxide simultaneously with the oxidation of phenolic compounds. BPx belong to polyfunctional enzymes, its activity is altered both in ontogenesis and under the influence of stress factors of various origins, which is due to conformational changes, posttranslational modifications, induction of enzyme synthesis and the emergence of new molecular forms (Passardi et al., 2005; Kim et al., 2008). Peroxidases catalyze crosslinking of cell wall components such as extensins, phenolic substances, and polysaccharides. Such strengthening of the cell wall can act as a mechanical barrier against the penetration of insects and pathogens (Wu et al., 1997). Peroxidases play an important role in protecting against insect attacks, and their activity has been reported to increase in response to herbivore or injury (Seliutina et al., 2020; de Lima Toledo et al., 2021). Peroxidases can enhance resistance to insect influence by oxidizing quinone, which can bind to proteins to reduce digestibility in insects (Dowd & Lagrimini, 1997). Being one of the most active enzymes in plant cells, catalases plays an important role in maintaining photosynthesis. CAT catalyzes the conversion of toxic hydrogen peroxide to water and molecular oxygen. The enzyme is localized in peroxisomes, where it removed H_2O_2 that was formed during photorespiration or during b-oxidation of fatty acids in glyoxysomes. Despite their restricted location, catalases play an important role in protecting plants from oxidative stress, since H_2O_2 can easily diffuse through membranes (Gill et al., 2010; Zhao et al., 2016).

Proteins are important for herbivorous insects, as they are directly related to the physiological function of insects (Le Gall et al., 2014; Deans et al., 2016). To date, there is little information confirming the difference in plant protein profiles in different species of the genus *Aesculus* during the colonization of their leaves by the Ohrid miner. In addition to being important as a nutrient for insects, soluble proteins contain a large number of enzymes, including those related to the antioxidant system of plant defense against stress. Therefore, studying the content of soluble proteins can contribute to a better understanding of the plant-insect interaction.

Currently, information on induced biochemical resistance of the genus *Aesculus* species in response to the mining moth attacks was mainly derived from the studies on the response of secondary metabolites to foliar damage by phytophagous insects (Mierziak et al., 2014; Oszmiański et al., 2015; Pastierovič et al., 2024). In addition, current research on plant-insect interactions has mainly focused on genomics and proteomics (Barbero & Maffei, 2023). Data on enzymatic responses as defense mechanism in *Aesculus* species that differ in their resistance to miner damage are still limited. The study hypothesized that induced responses of antioxidant enzymes and proteins are essential as component of the antioxidant defense system in the development of interactions between *Aesculus* and herbivorous insect *C. ohridella*.

This study aimed to assess the effectiveness of the antioxidant system and its species-specific response in *Aesculus* species with distinct resistance towards *C. ohridella* attacks. This study was focused on the induction of oxidative enzymes (peroxidase and catalase), highly soluble protein concentration in leaves of 15-year-old representatives of six *Aesculus* species.

Material and Methods

Collection of the leaves and sample preparation

As the research objects, we used following species of the genus *Aesculus*: horse chestnut *Aesculus hippocastanum* Linnaeus (*Hippocastanum* K. Koch. section); Ohio buckeye *A. glabra* Willdenow 1809, yellow buckeye *A. flava* Solander 1788; red buckeye or firecracker plant *A. pavia* Linnaeus 1753 (*Pavia* K. Koch. section); bottlebrush buckeye *Ae. parviflora* Walter 1788 (*Macrothyrsus* K. Koch. section), red horse chestnut *Aesculus* × *carnea* Hayne 1818 were introduced in 2008 within territory of the Botanical Garden of Oles Honchar Dnipro National University (Oles Honchar DNU) (48°26'07"N, 35°02'34"E; Dnipro city, Steppe zone of Ukraine).

C. ohridella-caused degree of leaf blade damage in horse chestnut was evaluated visually with a modified scale proposed by Zerova et al. (2007). The greatest resistance (by the area of leaf damage by insects) was observed in *A. parviflora*, *A.* × *carnea* and *A. flava* (0, 2, 5%, respectively), and the lowest in *A. hippocastanum* (52,5%). Average values of *C. ohridella*-caused leaf damage were determined in *A. pavia* and *A. glabra* (15% and 25%, respectively). To determine the reason for these differences, we analyzed the enzymatic antioxidant defense system.

We sampled seven leaves, each non-infested and infested by *C. ohridella* from each tree in the first decade of July 2023. Isolation and determination of the content of soluble proteins were carried out with biochemical analyses. The plant material was homogenized in 0.05 M tris/HCl buffer, pH 7.4 (ratio 1:20 (m/v)) with 0.5% polyvinylpyrrolidone (PVP) in a porcelain mortar in an ice bath. Extracts were centrifugated at 14,000 rpm for 15 min at 4 °C, and the supernatants were immediately used in assays of enzymes and proteins.

Biochemical analysis

The protein amount in a sample was determined according to Bradford protein assay (1976). The total soluble protein content was determined from the standard curve plotted by using bovine serum albumin (Serva, USA) as the standard. The soluble protein content of the samples was expressed in mg/g FW (fresh weight) of the plant material.

Benzidine peroxidase activity was detected with benzidine method at 490 nm, when benzidine was used as substrate according to Gregory (1966). The activity was calculated at 1-min time interval, in which the maximum reaction rate was observed [3]. The results were expressed in U g^{-1} FW * min^{-1}. To detect peroxidase isoforms, protein extracts were applied and separated in an IEF gel (5% polyacrylamide gel, 1 mm thick, pH 3.0–6.0; Amersham Biosciences) prepared in accordance with manufacturer's instructions. Isoelectric focusing was performed during 1.5 hours in the LKB 2117 system Ultrophor (LKB, Bromma, Sweden). Measurements of pH values were performed directly on a gel at 1-cm intervals using a microelectrode (LKB 2117-111 Multiphor Surface Electrodes) at +8 °C. Analysis of peroxidase isoform activity was carried out with the method of benzidine staining as described Guikema and Shermen (1980). The resulting gels were analyzed using ID Phoretics software. Isoelectric point (pI) values of the isoforms were calculated with calibration curve.

Guaiacol peroxidase activity was measured according to the method of Gechev et al. (2002) based on oxidation of guaiacol in the presence of hydrogen peroxide by the increase in absorbance at 470 nm during 2 min. Catalase activity was detected according to the method of Góth (1991) by measuring H_2O_2 which is capable of forming a stable complex with ammonium molybdate.

Statistical analysis

Repeated measures analysis of variances (ANOVA) (n = 5–7) was used to assess statistical differences between the species analyzed using Statistica version 8, StatSoft, Inc. USA. Tukey's test was used to determine the significant difference between the control and damaged groups of leaves. Data with P ≤0.05 were found to be statistically significant. The procedure of Variance Components Analysis was used to estimate the contribution of species-specificity of the impact, influence of the presence/absence of leaf mines, and interaction between the influence of species-specificity and presence/absence of leaf mines to the variability of biochemical parameters in *Aesculus* species.

Results

The species-specific traits determined 78.3% variability in protein content (F = 3509, P <0.001) (Fig. 1).

Being a response to the leafminer-caused damage, the overall trend of variability could explain 6.7% variation in protein content (F = 274, P <0.001). A species-specific response to the leafminer damage could explain 14.6% variation in protein content (F = 301, P <0.001) (Fig. 1). The general trend of response to the leafminer-caused damage consists in reducing protein content. Response patterns of *A. hippocastanum* and *A.* × *carnea* deviated from the general response trend. Protein content in these species increased in response to the leafminer damage; this was especially noticeable in *Ae. hippocastanum* (by 47.2%), while in *Ae.* × *carnea* protein content increased by 9% (Table 1).

The greatest decrease in protein content was observed in *A. flava* (by 63.5%) and *A. pavia* (by 32.1%). Species-specific traits determined 89.4% variability of BPx activity (F = 1864, P <0.001) (Fig. 1). Being a response to the insect-caused mining, the overall trend of variability could explain 0.7% variation in BPx activity (F = 6.1, P = 0.016). The general trend of response to the insect-caused mining consists in reducing BPx activity. A species-specific response to the leafminer-caused damage could explain 9.2% variation in BPx activity (F = 92, P <0.001). The responses of sweet buckeye (*A. flava*) and horse chestnut (*A. hippocastanum*) deviated from the general response trend, activity in their leaves increased by 61.4% and 29.6%, respectively, in response to the moth mining activity (Table 1). The lowest decrease (by 6.7%) in BPx activity was recorded in *A.* × *carnea*. Nearly the same level of inhibition of BPx activity in the presence of the horse-chestnut leaf miner larvae on the leaves was observed in

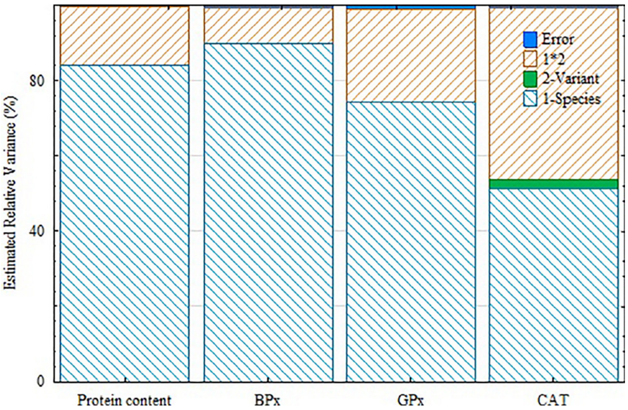

Fig. 1. Assessment of the influence of species-specificity and presence of leaf mines on variability of biochemical parameters in *Aesculus* species. Y-axis – % of variation explained by the influence of: 1 – species-specificity of the impact, 2 – influence of the presence/absence of leaf mines, 1*2 – interaction between the influence of species-specificity and presence/absence of leaf mines (species-specificity of the influence of presence/absence of leaf mines); Error – variability that is not described in the model: errors or variation, which is explained by factors other than ones considered in the model. X-axis – protein content, mg/g (R_{adj}^2 = 0.99, F = 1756, P< 0.001); BPx activity, U g^{-1} FW * min^{-1} (R_{adj}^2 = 0.99, F = 889, P <0.001); GPx activity, mM TGv g^{-1} FW * min^{-1}(R_{adj}^2 = 0.98, F = 729, P <0.001); CAT activity, μmol H_2O_2 g^{-1} FW * min^{-1}(R_{adj}^2 = 0.99, F = 1043, P <0.001)

representatives of *A. pavia* and *A. glabra* (by 27.5 and 29.3%, respectively).

Species-specific traits determined 73.3% variability of GPx activity (F = 1401, P <0.001). Being a response to the leafminer-caused damage, the overall trend of variability could explain 1.2% variation in GPx activity (F = 11.5, P <0.001). The general trend of response to the leafminer-caused damage consists in reducing GPx activity. A species-specific response to such damage could explain 24.6% variation in GPx activity (F = 202, P <0.001) (Fig. 1). Response patterns of *Ae. pavia* and *Ae.* × *carnea* deviated from the general response trend. GPx activity in these species increased in response to the leafminer damage by 74.6 and 16.8%, respectively. No statistically probable response to the leafminer damage was established for *A. glabra* (planned comparison F = 0.18, P = 0.67) (Table 1).

Analysis of IEF profiles in control BPx samples revealed from 2 (*A. hippocastanum*) up to 6 (*A. pavia*) acidic isoforms (Fig. 2a–f).

Relative content of the main form of the enzyme with pI 4.29 in leaves of the insect-resistant species *A. parviflora* was 46.3% (Fig. 2e). In *Ae. glabra*, isoperoxidases with pI 4.25 and 4.40 showed a tendency to increase activity under *C. ohridella* impact (Fig. 2a). In response to the leafminer-caused damage of *A. flava* leaves, an increase in the activity of isoform of pI 4.17 (by 34.8%) was recorded (P <0.05). A decrease in activity was showed in zone with pI value 4.25 (by 21%; P < 0.05) (Fig. 2b). The peroxidase system of *A. pavia* showed a slight decrease in the activity of isoforms, with the exception of the minor enzyme with pI 4.55, the activity of which was increased by 2.1 times (P < 0.05) (Fig. 2c). No significant differences were detected in BPx composition of *A.* × *carnea* leaves (Fig. 2d). In the presence of the moth mines on *A. hippocastanum* leaves, isoperoxidase activity with pI 4.25 was increased by 7.5%, and with pI 4.03 it was reduced by 15.3% (P < 0.05) (Fig. 2f).

Species-specific traits determined 51,2% variability of CAT activity (F = 1650, P <0.001). As a response to the leafminer-caused damage, the overall trend of variability could explain 2.4% variation in CAT activity (F = 673, P <0.001) (Fig. 1). The general trend of response to the leafminer-caused damage consists in reducing CAT activity. A species-specific response to the leafminer-caused damage could explain 46.8% variation in CAT activity (F = 510, P <0.001). Response patterns of *A. glabra* and

Table 1. Descriptive statistics of biochemical traits in *Aesculus* species

Species name	Variant	Protein content, mg/g	BPx activity, U g^{-1} FW * min^{-1}	GPx activity, mM TGv g^{-1} FW * min^{-1}	CAT activity, μmol H_2O_2 g^{-1} FW * min^{-1}
A. hippocastanum	ni	4.01±0.10a	71.71±2.83a	1.81±0.014a	4.54±0.007a
	i	5.90±0.15b	92.91±3.16b	0.74±0.007b	1.64±0.009b
A. pavia	ni	6.26±0.02a	61.43±8.43a	0.71±0.004a	2.95±0.005a
	i	4.25±0.03b	44.52±4.46b	1.24±0.003b	3.11±0.007b
A. × *carnea*	ni	9.11±0.19a	202.40±10.25a	1.84±0.001a	4.59±0.027a
	i	9.93±0.10b	188.78±2.73b	2.15±0.021b	3.64±0.005b
A. glabra	ni	4.07±0.07a	105.18±4.15a	0.70±0.002a	4.19±0.015a
	i	3.78±0.12b	74.42±1.98b	0.72±0.001a	4.85±0.038b
A. parviflora	ni	17.94±0.34	242.00±15.85	2.70±0.009	1.46±0.013
A. flava	ni	10.30±0.04a	96.56±3.28 a	1.46±0.001a	3.61±0.001a
	i	3.76±0.02b	155.87±5.92b	1.35±0.001b	3.43±0.001b

Notes: the values marked with different letters (a, b) are significantly different according to Tukey t-test P <0.05; non-infested leaves (*ni*); infested leaves (*i*) by *C. ohridella*; (mean ± SD, n = 7, N = 84)

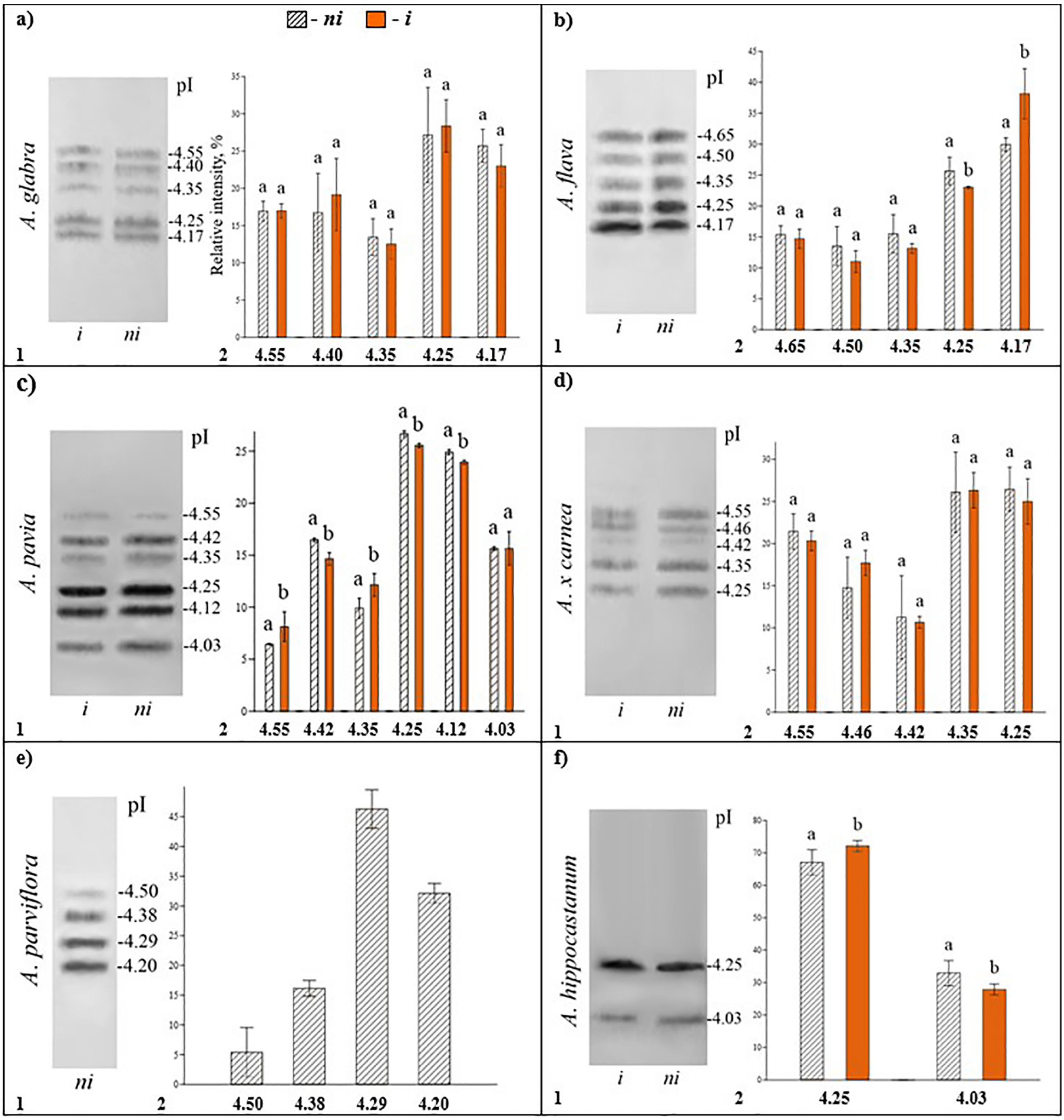

Fig. 2. Analyses of IEF performed in the range of pH values 3,5–6.0 (1) in the representatives of *Aesculus* species. The output of densitometric analysis is shown as relative content (2); *ni*: non-infected; *i*: infected leaves; the values marked with different letters (a, b) are significantly different according to Tukey *t*-test $P < 0.05$; (mean ± SD; n = 3)

A. pavia deviated from the general response trend. CAT activity in these species increased in response to the leafminer damage by 15.8% and 5.4%, respectively (Table 1).

Discussion

One of the advantages of successful introduction of plant species is its low sensitivity to pests and diseases (Jansone et al., 2023). Introduced *Aesculus* species *A. parviflora*, *A. pavia*, *A. flava*, *A. glabra* and *A. x carnea* growing in DNU Botanical Garden (as ecologically favorable area of the city) are the only *Aesculus* plantation in the region. In addition to decorative application, they also hold a certain forest potential, since their species composition is limited in arid steppe climates. Therefore, there was a need to study the level of their resistance to *C. ohridella* for the purpose of further implementation both in the planting of populated areas and in the forestry of the Steppe zone of Ukraine. In this regard, the authors for the first time conducted a comparative study of the enzymatic antioxidant system of leaf protection of various species *Aesculus* against the influence of the horse-chestnut leaf miner.

The species studied were arranged according to the level of their resistance to the miner attacks as follows (in order of descending the resistance level): *Ae. parviflora* (0%) > *Ae. carnea* (2%) > *Ae. flava* (5%) > *Ae. pavia* (15%) > *Ae. glabra* (25%) > *Ae. hippocastanum* (52.5%).

Alterations in gene expression under the horse-chestnut leaf miner infestation resulted in qualitative and quantitative changes in proteins responsible for signal transmission and oxidative defense in plants (War et al., 2018). Any imbalance in digestion during consuming plant proteins, such as, for example, proteinase inhibitors, affects the insect physiology. We found significant species-specific variability in protein content. As our output showed, the greatest concentration of highly soluble proteins was detected in leaves of a highly resistant species *A. parviflora*. In comparison with other species studied, the level of its content was exceeded by 1.7 (in *A. flava*) – 4.5 (in *A. hippocastanum*) times. Principial trend of the response to the leafminer-caused damage consists in reducing the protein content in three species (*A. glabra*, *A. flava* and *A. pavia*). It is consistent with the literature data indicating that a decrease in the content of total soluble protein is the principial pattern of the influence of chewing insects (Singh et al., 2013). The paper of Zogli et al. (2020) reported that *Schizaphis graminum*, as a result of feeding on the leaves of *Panicum virgatum* L., suppresses the accumulation of proteins involved in photosynthesis. As the authors note, such response of plants to insect damage may be important for the participation of enzymes in the biosynthesis of protective secondary metabolites. In addition, direct defense factors such as protease inhibitors and protective peptides have been reported in response to leaf feeding by insects, and other toxic proteins that reduce leaf digestion by phytophages (Emebiri et al., 2016; Meriño-Cabrera et al., 2018). This indicates the protective nature of such a reaction, thus preserving the vital potential of cells necessary for their further recovery. The most significant decrease in this parameter in our study was observed in the leaves of *A. flava*. However, the response of *Aesculus x carnea* and *A. hippocastanum* deviated from general trend; protein content in which increased in response to mining and, especially, in *A. hippocastanum*.

Taking into account that the introduced *Aesculus* species differed significantly in the level of leaf damage by the leaf miner, a comparative study of biochemical parameters associated with plant protection against damaging effects of the chestnut moth miner *C. ohridella* was conducted. Our studies of the antioxidant status of non-infested leaves revealed higher levels of benzidine peroxidase in the species *A. parviflora*, *A. x carnea*, *A. flava* and *A. glabra* compared to non-resistant *A. hippocastanum*. Better antioxidant activity in leaves of *A. flava* compared to *A. hippocastanum* was also shown by Štajner et al. (2014). High GPx activity was inherent for *A. parviflora* and *A. x carnea*, and the smallest for *A. glabra*. Catalase showed the highest level of activity in non-infested leaves of *A. x carnea* and *A. hippocastanum*.

Based on the results obtained, a wide range of interspecific variability in the activity of enzymes and the content of highly soluble proteins in the leaves of the studied species was established, which may indicate a presence of a variety of plant defense mechanisms in *Aesculus* species against the stress. In this study, *Aesculus* species exhibited multidirectional response to BPx expression under biotic stress (*C. ohridella* attacks). Increased BPx activity was recorded in the following species: *A. hippocastanum* and *A. flava* against the background of a decrease in GPx and CAT activities. Another response mechanism to the presence of mines on leaves was recorded in *A. pavia* and *A. glabra*: a decrease in BPx activity compensated by an increase in GPx I CAT activity. Moreover, very significant increase (by 74.6%) in GPx levels and a slight increase in CAT activity (5.4%) were found in *A. pavia*. On the contrary, catalase activity in *A. glabra* was higher, and guaiacol peroxidase activity remained almost at the control level. Hybrid *A. × carnea* showed only an increase in GPx activity, while BPx and CAT levels were reduced, especially CAT; it can indicate low ROS levels in the leaf tissue.

The formation of the host plant's response to an insect attack was closely related to the processes of secondary metabolism, which was reflected as alterations in activity of enzymes. The plants with a greater ability to produce antioxidants and defense enzymes (SOD, POD, PPO, CAT, and PAL) were considered to be more resistant (Walczak et al., 2019). Elevated levels of polyphenolic compounds such as (-)-epicatechin and, especially, polymeric procyanidins found in *A. glabra*, *A. parviflora* and *A. × carnea* may be explained by lower sensitivity of these species to *C. ohridella* infestation (Oszmiański et al., 2015). In this study, young *A. glabra* plants were found to be more sensitive to the horse-chestnut leaf miner compared to *A. × carnea* and *A. pavia*; it correlated with reduced BPx activity and a tendency to increase GPx activity in the leaves of this species. While in *A. × carnea* and *A. pavia* an increase in GPx activity was detected.

Being highly resistant species, *A. parviflora* was characterized by a high constitutive level of activity of BPx, GPx and soluble proteins in leaves compared to unresistant *A. hippocastanum* and moderately resistant species *A. glabra* and *A. pavia*. Hence, in comparison with other *Aesculus* species, BPx and GPx activities in small-flowered horse chestnut were 1.2–3.9 and 1.5–3.9 times higher, respectively. We can assume that such high values of peroxidase activity may contribute to the phenomenon of *A. parviflora* resistance due to the enhanced lignification process in tissues, as well as an increased content of secondary metabolites, in the synthesis of which peroxidases are involved (Pandey et al. 2017).

Enzymes occur often in many isoforms and are involved in synthesis of defense substances (Bijak & Lachowicz, 2021; Wielkopolan & Obrępalska-Stęplowska, 2016; Zhu et al., 2014). From the results presented here, a quantitative redistribution of activity between different molecular forms of benzidine peroxidase can be considered the principal pattern of alterations in benzidine peroxidase expression caused by the damage of *Aesculus* leaves by *C. ohridella*. The results showed a clear species-specificity of BPx IEF profiles, which differed both in the number and relative intensity of isoperoxidases with the same isoelectric point value.

The lowest number of BPx components (2 isoforms) was found in 15-year-old *A. hippocastanum* trees, while six isoperoxidases were found in 50-year-old horse chestnut trees in our previous study (Shupranova et al. 2019). In IEF spectra of BPx samples of other species, four (*A. parviflora*), five (*A. flava*, *A. glabra*, *A.* × *carnea*) and six (*A pavia*) acidic isoforms were detected in a fairly narrow range of pI values: 4.03–4.65. The dominant isoperoxidase in the leaves of all species was active zones with pI 4.17 and 4.25 (*A. flava*), 4.29 (*A. parviflora*), and 4.25 (*A. glabra*, *A.* × *carnea*, *A. hippocastanum* and *A. pavia*). In total, 14 molecular forms were identified in the spectra of benzidine peroxidase from the leaves of six *Aesculus* species. The most active peroxidase isoforms were localized in the range pI 4.12–4.35. Induction of isoperoxidases with pH levels of 4.25, 4.55 and 4.17 was detected in the leaves of the species *A. hippocastanum*, *A. pavia* and *A. flava*. This may indicate the involvement of acid isoperoxidases in the response of plant defense against stress. The role of individual peroxidase isoforms in protection against pathogens was confirmed in a study by Jang et al. (2004). *A. glabra* and *A.* × *carnea* showed no significant differences in BP IEF-profiles between non-infested and infested leaves. The data obtained demonstrated that the genus *Aesculus* species specifically respond to *C. ohridella*-caused mechanical damage to their leaves because of quantitative changes in the level of activity of individual molecular forms of peroxidase.

Catalase activity was also associated with the plant response to herbivorous insect infestation. During the research, we registered varying degrees of CAT activity inhibition in three of the five species **(***A. hippocastanum*, *A.* × *carnea* and *A. flava***)**. Heng-Moss et al. (2004) also found a decrease in catalase activity as a response to feeding of *Blissus occiduus* Barber on a sensitive buffalo grass variety, while a resistant variety retained the activity of this enzyme. No alterations in catalase activity of wheat varieties were reported in response to feeding of *Eurygaster integriceps* Puton (Rangasamy et al., 2009). A decrease in catalase activity was found during *C. ohridella* feeding on the leaves of *A. hippocastanum* in our previous paper (Shupranova et al., 2019). This enzyme is a peroxidase competitor because both of them use the same substrate (H_2O_2). Increased CAT activity has been reported after inoculation of tobacco plants with *Erysiphe cichoracearum*. This enzyme can act consistently with other antioxidant enzymes to neutralize pest-caused ROS damage, and thus may enhance the plant resistance. Hence, a significant decrease in catalase activity associated with very high peroxidase activity was noted (Buonario & Montalbini, 1993); the same pattern was recorded during our study in *A. flava*, where significant increase in BPx activity was accompanied by 50% decrease in catalase levels. In general, catalase activity increased only in *A. glabra* under the influence of *C. ohridella*, and the principal trend concerned a decrease in CAT activity of all other *Aesculus* species studied, and, especially, in *A. hippocastanum*. It may indicate excessive production of hydrogen peroxide in the leaf tissues. A decrease in catalase concentration is not the only possible cause for an increase in hydrogen peroxide concentration in plant tissues. Particularly, this effect may be attributable to increased activity of superoxide dismutase (SOD) that catalyzes the conversion of superoxide radical into hydrogen peroxide, which may contribute to hydrogen peroxide accumulation, especially in the setting of catalase inhibition.

Our research had shown that high tolerance of *A. parviflora*, *Aesculus x carnea* and *A. flava* to the moths influence primarily provide constitutively high levels of leaf peroxidase activity, which gives them advantages in protecting against phytophage attacks. This supports our hypothesis about the importance of the enzymatic defense system in interaction *Aesculus C. ohridella*, which will provide beneficial information for breeding and biotechnology programs.

Conclusion

The data obtained in this study suggested that biochemical defense profiles distinguish clearly between resistant *Aesculus* species of North American origin and their sensitive Balkan analogue, *A. hippocastanum*. At the biochemical level, the results obtained in the study of plant/phytophage relationships are important for understanding the nature of *Aesculus* species adaptation to their colonization by *C. ohridella*. High stability of *A. parviflora*, *A. flava* and *A.* × *carnea* derived from the effective functioning of the enzymatic antioxidant defense system, which ensures successful existence of these species both in the novel environment (Steppe zone of Ukraine) and in the conditions of *C. ohridella* attacks. These species can be recommended to be used in introduction into green spaces of urbanized environment and forestry, as well as used in breeding and biotechnological programs to improve plant resistance to phytophagous pests.

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