Research paper

Effects of *Parectopa robiniella* Clemens (Gracillariidae) on the Antioxidant Defense System of *Robinia pseudoacacia* L. Trees of Different Ages

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【Summary】

Our study aimed to evaluate the effectiveness of the enzyme defense system of the assimilation apparatus of *Robinia pseudoacacia* L. (black locust) against damage caused by invasive phytophages. The study focused on trees in plantations clustered in 3 age groups: I, II, and III corresponding to the age ranges of 5-10, 15-25, and 50-70 yrs. The study discovered an increase in peroxidase activity with the progression of tree age. Damaged leaves of plants from group III showed significantly greater benzidine- and guaiacol-peroxidase activity (by 68.6 and 180%, respectively) compared to undamaged leaves of the same group. Guaiacol-peroxidase activity was found to have significantly increased in trees attacked by *Parectopa robiniella* in groups I and III (by 15.5 and 180%, respectively), but only increased enzyme activity was recorded in trees of group II. Levels of peroxidase activity in leaves undamaged by insects (intact) were similar in young trees (groups I and II) compared to trees in group III in plantations which initially had significantly higher activity. This was confirmed through study of the isoenzyme composition of peroxidase in which leaves of plants in group III showed more activity of molecular forms of the enzyme. In the analyzed age groups of trees affected by *P. robiniella*, catalase activity did not significantly differ compared to that of undamaged samples. In conclusion, a peroxidase system can be considered the main defense system of black locust.

Key words: *Parectopa robiniella* Clemens, *Robinia pseudoacacia* L., benzidine-peroxidase, guaiacol-peroxidase, catalase.

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研究報告

刺槐指狀潛葉蛾(細蛾科)對不同樹齡刺槐抗氧化防禦系統的影響

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摘 要

本研究目的在評估刺槐 (*Robinia pseudoacacia* L.) 同化結構中酶防禦系統對抗入侵植食性昆蟲所造成損害的效能，以3個齡級（I、II、III分別為5-10、15-25和50-70年生）的人工林木為研究對象。結果發現過氧化酶活性隨著刺槐樹齡的增加而增強。與未受損的葉子相比，III齡級植株受損葉子的聯苯胺過氧化酶和癒創木酚過氧化酶活性較相同齡級植株未受損葉顯著增強（分別提高68.6%和180%）。在I和III齡級刺槐，癒創木酚過氧化酶活性在受到刺槐指狀潛葉蛾 (*Parectopa robiniella* Clemens) 危害下顯著增加15.5%和180%；但II齡級刺槐，僅記錄到癒創木酚過氧化酶活性有增加的趨勢。幼樹（I和II齡級）中未受昆蟲損害（完整）葉子的過氧化酶活性相似，相較之下III齡級刺槐一開始就具有顯著較高的過氧化酶活性表現。透過過氧化酶同功酶組成的研究，證實III齡級刺槐葉子具有更高酶活性表現。所有檢測齡級的刺槐，受刺槐指狀潛葉蛾危害葉片的過氧化氫酶活性與未受損的樣本之間並無顯著差異。結論，過氧化酶系統被認為是刺槐的主要防禦系統。

關鍵詞：刺槐指狀潛葉蛾、刺槐、聯苯胺過氧化酶、癒創木酚過氧化酶、過氧化氫酶。

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INTRODUCTION

In Ukraine, the species composition of steppe forests is limited by extreme edapho-climatic conditions. Introduction of plants to the forest is essential to improving the qualitative composition and productivity of forests by creating artificial forest phytocenoses which are more resistant to harmful abiotic and biotic factors than local ones (Mathakutha et al. 2019, Gorban et al. 2020, García et al. 2023). Black locust (*Robinia pseudoacacia* L., Fabaceae) is one of the most common and environmentally adaptable invasive tree species in the northern steppe zone of Ukraine. This species originates from eastern North America (Vítková et al. 2020, Guo et al. 2022). It is considered one of the most aggressive invasive newcomers in Central Europe due to its adaptability to drought, rapid vegetative spread, and nitrogen fixation, which are all indicators of plant invasiveness (Daehler 2003, Pyšek et al. 2012). This species also grows successfully in industrial areas of cities (Tzvetkova and Petkova 2015, Środek and Rahmonov 2022). Lazzaro et al. (2018)

noted that black locust reduces biodiversity mainly by reducing the amount of light reaching other plants under the canopy, as well as by changing microclimate or soil conditions (nitrification and acidification). Forest-typical oligotrophic and acidophilic associations are removed, and neutrophilic weed associations begin to predominate, thereby reducing plant diversity (Benesperi et al. 2012, Nicolescu 2020). Black locust plantations also perform many ecosystem services however, including reclamation, soil-protection, environment-formation, and ornamental functions, as well as being a beneficial honeybee and medicinal plant (Yang et al. 2010, Li et al. 2021).

The widespread distribution of leaf-mining insects is of great concern. Their significance among other phytophages has increased due to their high adaptive capacity to endure substantial levels of contamination, moisture deficits, the effects of insecticides, as well as having multiple generations per year (Tytar et al. 2022). The core of the modern invader complex includes distant invasive species originating from North America, Southeast Asia, and the Far East; among them, the locust digitate leaf miner (*Parectopa. robiniella* Clemens, 1863, Graciilariidae) was detected on *R. pseudoacacia* trees in all geographical areas of the country. The caterpillar of this moth feeds on leaf mesophyll and forms whitish complex-shaped mines, approximately 1.4 cm^2 in size. Among the factors that determine the econiches of the locust digitate leaf miner, the most significant are temperature-related, which contribute to the spread and naturalization of the phytophage.

In Ukraine, the appearance of this moth coincided with an increase in average annual temperatures (Tytar et al. 2022). Within its native range, *P. robiniella* acts as an oligophagous species damaging the leaves of several woody species of the family Fabaceae: *Robinia pseudoacacia* L., *Robinia hispida* L., *Robinia viscosa* Vent., *Amorpha fruticosa* L., *Desmodium* sp., *Galactia volubilis* (L.) Britt., and *Meibomia* sp. (Davis and De Prins 2011).

The growing body of information accumulated over recent years has required refining the concept of a “plant-insect” system as a simple interaction between 2 organisms (Mithöfer and Boland 2012). Interactions between plants and insects are complex and involve numerous factors. Leaf characteristics are closely related to plant responses and to environmental conditions, in particular, to attacks by invasive insects, and can provide important information about adaptation to stresses of biotic origin (Liu et al. 2017), as well as changes in environmental factors, particularly attack by herbivorous insects, which alter primary metabolism, including the photosynthesis rate, nitrogen uptake, protein content, antioxidant activity, and tree lifespan. Damage to leaves reduces the photosynthetic surface area, threatening the entire production process of the plant (Huang et al. 2016, Turfan et al. 2018). The defense mechanisms initiated by plants during invasion by phytophagous insects include development of specific morphological structures, building of constitutive physical barriers from callose, suberin, and lignin, and the synthesis of toxic secondary metabolites (phenols, alkaloids, quinones, etc.) and volatile compounds, as well as antioxidant enzymes. The most important plant defense is enhancing the synthesis of antioxidant enzymes and increasing their activity. These are often found in many isoforms and are involved in the synthesis of protective substances, or have a direct antimicrobial effect (Luhová et al. 2002, Zhu et al. 2014, Acevedo et al. 2015, Wielkopolan and Obrępalska-Stęplowska 2016). Understanding of morphological, inhibitory, and other barriers of plant immunogenetic systems is supplemented by knowledge from biochemistry, genetics, and molecular biology (Smith and Clement, 2012, Emebiri et al. 2017). The involvement of plant proteins in the development of resistance to pathogens is well-studied (McHale et al. 2006), while understanding of the fundamental mechanisms of plant immunity to insects is still being developed (Wang et al. 2013). The protective responses of plants to attack by phytophagous insects and pathogens are mostly similar at the molecular level of signal transmission and triggering response reactions. Jasmonic acid is thought to be responsible for regulating defense mechanisms against predominantly chewing insects (Ray et al. 2015). Much attention has been paid to study of responses of secondary metabolites (phenols, quinones, etc.) to leaf damage by phytophages, while understanding of the mechanism of response of defense-related enzyme systems to plant damage by miners is lacking (Paterska et al. 2017). In our previous work, we investigated the response of antioxidant enzymes in leaves to harmful insect miners in the environment of an industrial city (Shupranova et al. 2022). We are not aware of similar studies.

The objective of this study was to assess the efficiency of the enzyme defense system of the assimilation apparatus of the black locust against the action of the invasive moth *P. robiniella* in regard to the age structure of trees from 3 age groups in artificial plantations.

MATERIALS AND METHODS

Plantations of *Robinia pseudoacaci*a L. of different ages: I-young trees (up to 15 yrs old), II-undergrowth (15-25 yrs old), and III-parent trees planted at the time of the creation of the forest belt (50-70 yrs old) located in a forest protection strip along a ravine near Mayorka Village were chosen for this study.

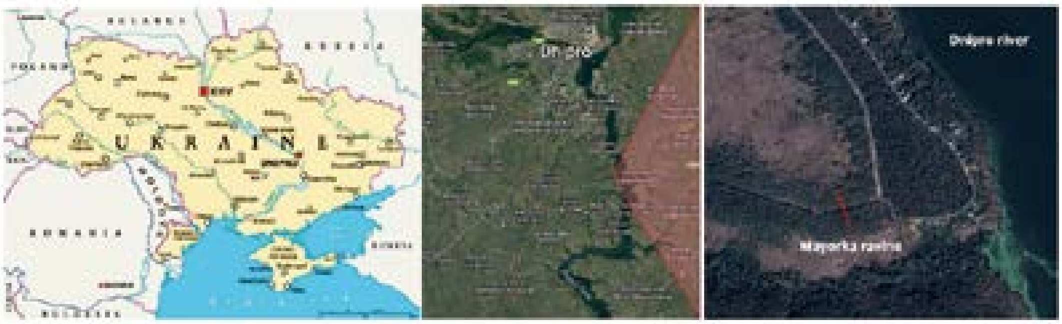


Fig. 1. Location of *Robinia pseudoacacia* L. plantations near Mayorka Village.

Mayorka Village is located on the right bank of the Dnieper River in the Dnipro District of Dnipropetrovsk Oblast (Fig. 1).

According to monitoring studies, average levels of infestation were 0.13±0.009 in group I, 0.08±0.006 in group II, and 0.05±0.003 in group III (calculated in fractions of a unit). Therefore, *P. robiniella* mainly forms mines on the leaves of young trees (up to 15 yrs old). The research was conducted in 2022 in Mayorka Village in Dnipropetrovsk Oblast (48º26'N, 35º16'E). The climate in this area is moderately continental with hot and dry summers and frequent downpours. According to long-term data, the dominant winds in Mayorka Village are northerly, north-easterly, and westerly. The area of Mayorka Village is characterized by the predominance of ordinary chernozem and meadow-chernozem soils; swamp-meadow and swamp soils are less common. Ordinary chernozems have a neutral reaction of the medium, with a humus content ranging 4.3-6%. One of the features of the climate in the area is significant fluctuations of weather conditions from one year to another. Hot dry winds blow often, and moderately wet years alternate with sharply dry ones.

Five leaves were taken from medium formations in the lower third of the southern exposure crowns of 7 *R. pseudoacacia* trees from each plot (an annual vegetative increment) in dry, clear weather. In total, 21 trees were explored.

Leaves were washed with water and immediately used for enzyme extraction. To isolate the enzyme preparation, black locust leaves (0.3 g) were homogenized in 6 ml of 0.05 M Tris-HCl buffer, pH 7.4, with 0.5% polyvinylpyrrolidone (PVP). Extraction was performed at +4 °C for 1 h and centrifuged for 15 min at 14,000 rpm. The supernatant was collected to determine the activity and isoenzyme composition of benzidine-peroxidase (BP), guaiacol-peroxidase (GP), and catalase (CAT).

BP activity (EC 1.11.1.7) was measured at 490 nm in a reaction mixture (0.8 mL Na-acetic buffer, pH 5.4; 1 mL of benzidine solution and 0.2 mL of enzyme preparation) after adding 1% H_2O_2. Activity was calculated within a 1-min time interval, and the maximum reaction rate was observed (Gregory 1966). The result was expressed in absorbance units g^{-1} of raw material*min.

The isoenzyme composition of BP was determined by isoelectric focusing (IEF) in a 5% horizontal polyacrylamide gel (PAAG) using Ultraphor (LKB, Bromma, Sweden), pH range 3.5-6.5, at +10°C. A benzidine method was used to detect enzymatic activity in PAAG. The stained gels were scanned and analyzed using 1D Phoretix software.

To determine GP activity (EC 1.11.1.7), guaiacol was used as a hydrogen donor, and hydrogen peroxide was used as a substrate. The incubation medium consisted of 50 mm Tris-HCI buffer (pH 5.4), 2.6 mM hydrogen peroxide, and 21.5 mM guaiacol. The observation period was 2 min. Activity was determined by the formation of a reaction product, tetraguaiacol (TG), due to an increase in the optical density at 470 nm. The amount of TG was calculated by taking into account the extinction coefficient $\varepsilon = 0.0266\ \mu M^{-1}\ cm^{-1}$). Activity was expressed in μM of TG formed per mg of protein for 2 min (μM of TG (mg of protein)$^{-1}$). CAT (EC 1.11.1.6) activity was evaluated according to Goth (1991), and results were expressed in $\mu M\ H_2O_2\ (min^{*}g)^{-1}$ of fresh leaves. The concentration of soluble proteins was determined using the Bradford protein assay (1976) and was expressed in mg g^{-1} of raw material. Five replicates of the protein and enzyme assays were conducted.

Statistical analysis

Results of the study on enzyme activity were presented as the mean ± standard deviation (SD). After testing the data for normality, differences in variables of enzyme activity and protein content between the different tree age groups were tested using a univariate analysis of variance (ANOVA) with Statistica vers. 8 package (StatSoft, Hamburg Germany). Tukey's honest significant difference (HSD) test was used to test differences among sample means for significance. Significant statistical differences were established using Tukey's HSD at $p < 0.05$. Numbers of replicates in the protein and enzyme assays were 5 biological and 3 analytical. Undamaged leaves were used as a control.

RESULTS

Detailed analysis of the effect of damage caused by *P. robiniella* on the biochemistry of black locust leaves is presented in Tables 1 and 2. Trees of the 3 groups responded differently to phytophage attacks. An increase in the protein content in leaves of black locust trees in group I by 7% and a decrease of 9.1% in group II ($p < 0.001$) with feeding by caterpillars were found (Table 1). There was no difference in protein contents between the reference and damaged leaves in group III (Table 3). Figure 2 confirms this result; here, values of the protein content in leaves of black locust trees of group III were far from values of groups I and II. The within-group coefficient of variation was low (0.4-1.5%), and the between-group coefficient of variation was 17.1%. Significant differences in mean values of BP activity were not found between the reference and experimental samples of the black locust trees in groups I and II (Table 3).

An increase in enzyme activity was observed. Older trees showed a significant difference in this parameter between intact leaves and leaves damaged by phytophage, namely an increase in BP activity by 68.6% (Table 1). The variability of values was very low within age groups (1.2-2.6%), but was high between groups, amounting to 41.3% ($p < 0.001$).

It should be noted that the difference between the mean values of enzyme activity in reference leaves of trees of groups I and II was insignificant, while BP activity in the leaves of trees of the old forest belt was 52.5% greater compared to young trees, and under the phytophage action the difference between young and old trees was 2.3 times higher.

When analyzing the activity of guaiacol-dependent peroxidase in reference samples, it was found that GP activity increased with increasing tree age: GP activity in the leaves of trees in group II increased by 12.8% and increased significantly (2.7 times) in group III compared to young trees of group I (Table 2). Significant increases ($p < 0.001$) in GP activ-

Table 1. Descriptive statistics for the variation in protein concentration and benzidine-peroxidase activity as a function of age

Age group	Protein, mg g^{-1} FL, $F = 312892$, $p < 0.001$			Benzidine-peroxidase, U $(\text{min g})^{-1}$ FL, $F = 95407$, $p < 0.001$		
	Mean±SD n=5	Min	Max	Mean±SD	Min	Max
I ref	2.64±0.04	2.59	2.71	44.53±1.16	42.79	45.53
I **exp**	2.83±0.02	2.80	2.85	45.75±0.73	44.55	46.45
II ref	2.74±0.01	2.73	2.76	47.16±0.57	46.54	48.03
II **exp**	2.49±0.02	2.47	2.52	55.85±0.78	55.22	57.14
III ref	1.85±0.01	1.83	1.87	69.84±1.17	68.54	71.24
III exp	1.85±0.01	1.84	1.87	117.72±1.84	115.72	120.06
All groups	2.40±0.41	1.83	2.85	63.48±26.22	42.79	120.06

U, optical density; FL, fresh leaves; SD, standard deviation; I, II, III, age groups; ref, reference; **exp**, experimental (damaged leaves).

Table 2. Descriptive statistics of variations in enzyme activities as a function of age

Age group	Guaiacol-peroxidase, μmol TG (min*mg protein FL)$^{-1}$, $F = 81347$, $p < 0.001$			Catalase, μM H_2O_2 (min*g FL)$^{-1}$, $F = 9483$, $p < 0.001$		
	Mean ± SD, *n*=5	Min	Max	Mean ± SD	Min	Max
I ref	6.53±0.16	6.32	6.71	2.93±0.14	2.76	3.14
I **exp**	7.54±0.35	7.14	8.03	2.77±0.09	2.64	2.88
II ref	7.68±0.19	7.43	7.89	2.51±0.11	2.38	2.63
II **exp**	8.51±0.23	8.29	8.89	2.42±0.12	2.25	2.57
III ref	17.53±0.40	16.91	17.97	2.23±0.19	2.03	2.48
III **exp**	49.12±0.42	48.39	49.44	2.08±0.16	1.85	2.23
All groups	16.15±15.46	6.32	49.44	2.49±0.32	1.85	3.14

FL, fresh leaves; I, II, III, age groups; SD, standard deviation; ref, reference; **exp**, experiment (damaged leaves); TG, tetraguaiacol.

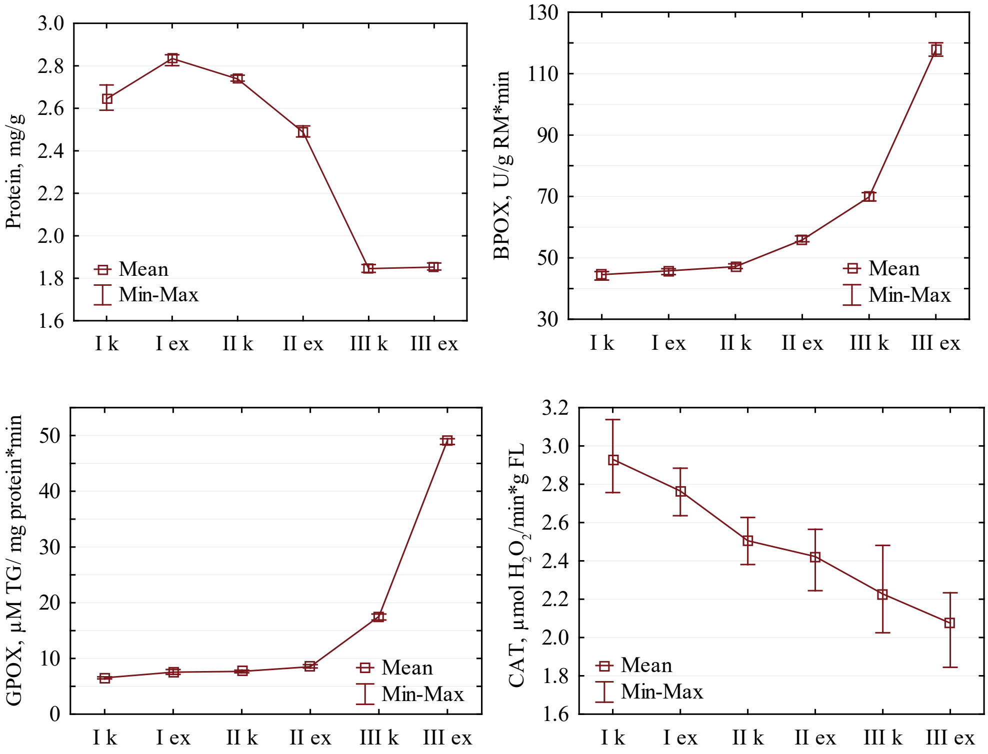


Fig. 2. Differences in enzyme activities and protein contents between damaged and intact leaves of *Robinia pseudoacacia* trees of different ages.

ity were recorded for groups I and especially III of 15.5 and 180%, respectively, compared to a reference (Fig. 2) in leaves of trees under the effect of the invasive caterpillar. The status of damaged leaves of trees of group II did not significantly differ compared to that of undamaged leaves (Table 3). There was only an increasing trend in GP activity. Significant variability in GP activity values was observed across trees of all age groups (95.7%).

Unlike peroxidases, CAT activity levels decreased with increasing tree age, both in the reference and damaged leaves. CAT activity in the leaves of trees of age groups II and III was significantly reduced by 14.3% and 23.9%, respectively, compared to trees of group I (reference) (Table 2). A similar pattern was found for damaged leaves: compared to group I, activity levels in black locust leaves in age groups II and III decreased by 12.6 and 29.0%, respectively. Within age groups, a decreasing trend in CAT activity in leaves

Table 3. ANOVA results: post-hoc Tukey's test of protein concentration and enzyme activities

Age group	I **exp**	II ref	II **exp**	III ref	III **exp**
Protein, mg g^{-1}					
I ref	*	*	*	*	*
I **exp**		*	*	*	*
II ref			*	*	*
II **exp**				*	*
III ref					ns
Benzidine-peroxidase, U (g FL*min)$^{-1}$					
I ref	ns	ns	*	*	*
I **exp**		ns	*	*	*
II ref			*	*	*
II **exp**				*	*
III ref					*
Guaiacol-peroxidase, μM TG (mg protein*min)$^{-1}$					
I ref	*	*	*	*	*
I **exp**		ns	*	*	*
II ref			ns	*	*
II **exp**				*	*
III ref					*
Catalase, μM H_2O_2 (min*g FL)$^{-1}$					
I ref	ns	*	*	*	*
I **exp**		ns	ns	*	*
II ref			ns	ns	*
II **exp**				ns	ns
III ref					ns

Ref, reference; **exp**, experiment; FL, fresh leaves; TG, tetraguaiacol; * significant at $p < 0.001$; ns, no significant difference between means at $\alpha = 0.05$.

of groups I and II in response to phytophage action (5.5 and 3.6%, respectively) was found (Fig. 2). A significant difference was observed only in leaves of trees of group III where CAT activity decreased by 6.7%. The variability of this parameter within a single age group was 3.8-8.5%, and the average value across all 3 age categories was 12.9%.

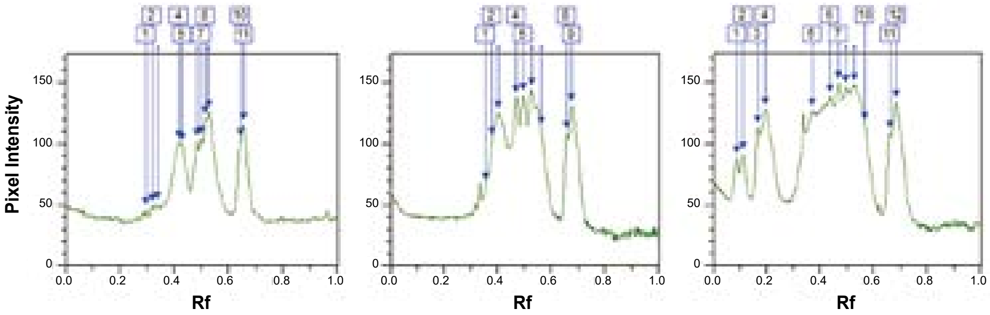
Analysis of the isoenzyme composition of BP in cells of the black locust leaves of different age groups showed variations in the spectrum of multiple forms of the enzyme from the normal physiological state (reference) (Fig. 3).

The number of components was 9 to 11 in the leaves of trees in the group I, 9 in group II, and 9-12 in the old forest belt; the intensity of the latter was greater than that of young trees.

DISCUSSION

In this study, a comparative biochemical analysis of leaves both undamaged and damaged by *P. robiniella* sampled from *R. pseudoacacia* trees in 3 age categories was performed. The uncertainty of the "plant-phytophage" interaction under natural conditions requires study of the protective mechanisms of different-aged *R. pseudoacacia* trees against attacks of invasive insects in urban environments. As a number of authors have shown, tree size and age influence variations in tree structure and function, affecting growth, germination, reproduction, and carbon accumulation (Duan et al. 2014). Activities of the antioxidant enzymes benzidine peroxidase, guaiacol peroxidase, and CAT, and the protein content were analyzed as resistance-related indicators. The degree of specificity for interactions in the "plant-phytophage" system has not yet been developed. However, this direction is rapidly developing (Every et al. 2005, Smith and Clement 2012, Seliutina et al. 2020).

To survive biotic stress, plants have developed complex mechanisms based on metabolite modifications, and gene and protein expressions (Luo et al. 2017). Changes in gene expressions under stress, including insect attacks, lead to qualitative and quantitative changes in proteins, which in turn play important roles in signal transmission and antioxidant defense in plants (Gulsen et al.



I II III

Age groups

Fig. 3. IEF profiles of benzidine-peroxidase (BP) in undamaged leaves of *Robinia pseudoacacia* trees of different ages; Rf, electrophoretic mobility.

2010, Usha and Jyothsna 2010, War et al. 2012, Koch et al. 2016). Protein and carbohydrates are particularly important nutrients for herbivorous insects because these substances stimulate their development and reproduction (Canbolat 2012). Our results showed that insects prefer eating protein when the total content of available macronutrients (carbohydrates and lipids) is low (Le Gall and Behmer 2014). Proteins provide the animal body with the amino acids needed to build new tissues, while carbohydrates are commonly used as a key source of energy required for enhancing protein biosynthesis. Our results showed that trees in groups I and II had a statistically significant difference in protein content (compared to the reference), but phytophage effects were ambiguous: the protein content increased in leaves of group I, but decreased in group II, while there was no difference between reference and experimental values in trees of the old forest belt. It can be assumed that protein levels in insect-damaged leaves decrease due to a slowing in the rate of protein synthesis, and the entire translation mechanism shifts towards the production of defense-related proteins. In addition, the decrease in protein levels in September may be caused by outflow to plant storage organs being a normal physiological process, but this process may be accelerated in phytophage-damaged leaves compared to reference ones. At the same time, a decrease in protein levels caused by stress can lead to the accumulation of free amino acids in cells, some of which serve as substrates for the synthesis of phenolic compounds as secondary metabolites involved in the defense of cellular structures. Alternatively, an increase in the intensity of protein synthesis under stress can lead to irreversible overruns of energetic resources (since protein synthesis is one of the most energy-demanding physiological processes), which will make the flow of reparation processes impossible after cessation of the action of the stressful factor (War et al. 2012).

With the discovery of signaling and protective roles of reactive oxygen species (ROS), much attention has been paid to oxidoreductases as regulators of their levels in the cell (Temple et al. 2005, Koch 2016). Among them, CAT and peroxidase are of particular interest as they are involved in the response to a wide range of environmental stresses and stimuli (Passardi et al. 2005). ROS were used as central early signals that contain information about the environment and regulate resistance to stress caused by insect feeding (Kerchev et al. 2012, Foyer et al. 2016, Zhao et al. 2016). All components of the antioxidant system (AOS) act mainly in a complex way, and changes in the concentration or activity of some antioxidants usually lead to certain alterations in others. Thus, our previous work (Shupranova et al. 2022) conducted in an urban environment showed that the resistance of *R. pseudoacacia* plants depended on the coordinated actions of antioxidant enzymes. Four main types of simultaneous changes in the activities of oxidative metabolism enzymes were registered in black locust leaves, reflecting the variety of its adaptive responses and allowing them to quickly rebuild their defense system against attacks of *P. robiniella* under various pollution conditions in an industrial city. In field conditions, in the absence of detrimental industrial and transport effects, only 1 type of simultaneous action of the studied enzymes was noted, namely an increase in the level of peroxidase activity and a decrease or tendency to reduce CAT activity. These compensatory roles of BP, GP, and CAT were often noted by researchers in various plants (Fernández-García et al. 2004, Chen et al. 2008, Zhao et al. 2016). Studies have shown that peroxidases and CAT behave differently during an attack by *P. robiniella* under natural conditions, which was typical for all age groups of black locust trees. Statistical data showed high variability of BP and GP activities (41.3 and 95.7%, respectively) across the studied age groups. This indicates that the enzymatic plasticity of *R. pseudoacacia* shows significant variable polymorphism. CAT is a competitor to peroxidase because both of them use the same substrate (hydrogen peroxide). Increased activity was reported after the inoculation of tobacco plants with *Erysiphe cichoracearum.* However, a significant decrease in CAT activity should be explained by very high peroxidase activity (Buonario and Montalbini 1993, Lebeda et al. 2001). CAT was noted to be in a stable state when the redox status of a cell changes, and therefore it is more stress-resistant compared to other components of the antioxidant system (Passardi et al. 2005). In our study, the response of enzymes to a phytophage attack showed that BP and GP activities significantly increased only in leaves of trees of age group III (by 68.6 and 180%, respectively). The isoenzyme profile of BP in leaves of older trees showed that these trees were physiologically better prepared for attacks by *P. robiniella* due to high activity levels of BP and GP isoforms. Reference leaves of black locust trees of age group III had significantly higher expressions of molecular forms of peroxidase compared to younger plants. This means that increased activity of ROS neutralizing peroxidases in more-tolerant plants of the old forest belt could more easily detoxify ROS emerging as a result of damage from invasive insects, and therefore such trees do not suffer from the detrimental effects of high ROS levels. A similar statement was indicated in the work of Smith et al. (2012). Increases in BP and GP activities indicated participation in plant defense against attack by invasive insects. BP is involved in the neutralization of ROS, and GP is involved in protecting of cell walls due to lignification and suberination to prevent phytophage attacks.

CONCLUSIONS

Results show that local induction of peroxidases is essential for H_2O_2 neutralization in response to caterpillar feeding and indicates the relevance of antioxidant enzymes in defense against damage by the moth *P. robiniella*. With increasing tree age, the defense system of *R. pseudoacacia* leaves is enhanced. This is accompanied by stimulation of peroxidase activity and a tendency to decrease catalase activity, which is considered a compensatory response to increased levels of benzidine- and guaiacol-peroxidase. Black locust plants of different ages attacked by invasive phytophagous insects showed a multifaceted response of the protein system: an increase, a decrease, and a lack of response. The fact that invaders preferred young leaves as a food source can be explained by lower BP and GP activities of young leaves compared to old ones. These results highlight the significance of peroxidase in the feeding of *P. robiniella* caterpillars, and this idea opens up prospects for enhancing plant resistance through increasing peroxidase expression.

LITERATURE CITED

Acevedo FE, Rivera-Vega LJ, Chung SH, Ray S, Felton GW. 2015. Cues from chewing insects－the intersection of DAMPs, HAMPs, MAMPs and effectors. Curr Opin Plant Biol 26:80-6. https://doi.org/10.1016/j.pbi.2015.05.029.

Benesperi R, Giuliani C, Zanetti S et al. 2012. Forest plant diversity is threatened by *Robinia pseudoacacia* (black-locust) invasion. Biodiver Conserv 21:3555-68. https://doi.org/10.1007/s10531-012-0380-5.

Bradford MM. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72:248-54. https://doi.org/10.1006/abio.1976.9999.

Buonario R, Montalbini P. 1993. Peroxidase, Superoxide dismutase and catalase activities in tobacco plants protected against *Erysiphe cichoracearum* by a necrotic strain of potato virus Y. Riv Pat Veg 3:23-31.

Canbolat Ö. 2012. Determination of potential nutritive value of exotic tree leaves in Turkey. Kafkas Univ. Vet Fak Derg 18(3):419-23. https://doi:10.9775/kvfd.2011.5584.

Chen JW, Cao KF. 2008. Changes in activities of antioxidative system and monoterpene and photochemical efficiency during seasonal leaf senescence in *Hevea brasiliensis* trees. Acta Physiol Plant 30(1):1-9.

Daehler CC. 2003. Performance comparisons of co-occurring native and alien invasive plants: implications for conservation and restoration. Ann Rev Ecol, Evol and Syst 34:183-211. https://doi.org/10.1146/annurev.ecol.sys.34.011802.132403.

Davis DR, De Prins J. 2011. Systematics and biology of the new genus *Macrosaccus* with descriptions of two new species (Lepidoptera, Gracillariidae). ZooKeys 98:29-82. https://doi.org/10.3897/zookeys.98.925.

Duan L, Liu H, Li X, Xiao J, Wang S. 2014. Multiple phytohormones and phytoalexins are involved in disease resistance to *Magnaporthe oryzae* invaded from roots in rice. Physiol Plant 152:486-500. https://doi.org/10.1111/ppl.12192.

Emebiri LC, Tan MK, El-Bouhssini M, Wildman O, Jighly A, Tadesse W, Ogbonnaya FC. 2017. QTL mapping identifies a major locus for resistance in wheat to Sunn pest (*Eurygaster integriceps*) feeding at the vegetative growth stage. Theor Appl Genet 130(2):309-18. https://dx.doi.org/10.1007/s00122-016-2812-1.

Every D, Sutton KH, Shewry PR, Tatham AS, Coolbear T. 2005. Specificity of action of an insect proteinase purified from wheat grain infested by the New Zealand wheat bug, *Nysius huttoni.* J Cereal Sci 42(2):185-91.

Fernández-García N, Carvajal M, Olmos E. 2004. Graft union formation in tomato plants: peroxidase and catalase involvement. Ann Bot 93(1):53-60. https://10.1093/aob/mch014.

Foyer CH, Rasool B, Davey JW, Hancock RD. 2016. Cross-tolerance to biotic and abiotic stresses in plants: a focus on resistance to aphid infestation. J Exp Bot 67:2025-37. https://doi:10.1093/jxb/erw079.

García P, Sanna M, García MF, Rodríguez GG, Cires E. 2023. Monitoring invasive alien plants dynamics: application in restored areas. Biologia https://doi.org/10.1007/s11756-023-01375-w.

Gorban V, Huslystyi A, Kotovych O, Yakovenko V. 2020. Changes in physical and chemical properties of *Calcic Chernozem* Affected by *Robinia pseudoacacia* and *Quercus robur* Plantings. Ekologia (Bratislava) 39(1):27-44. https://doi.org/10.2478/eko-2020-0003.

Goth L. (1991). A simple method for determination of serum catalase activity and revision of reference range. Clin Chim Acta 196:143-52. https://doi.org/10.1016/0009-8981(91)90067-m.

Gregory RPF. 1966. A rapid assay for peroxidase activity. Biochem J 101(3):582-3. https://doi.org/10.1042/bj1010582.

Gulsen O, Eickhoff T, Heng-Moss T, Shearman R et al. 2010. Characterization of peroxidase changes in resistant and susceptible warm season turfgrasses challenged by *Blis-*

sus occiduus. Arthropod Plant Interact 4:45-55. http://dx.doi. org/10.1007/s11829-010-9086-3.

Guo Q, Cao S, Dong L et al. 2022. Genetic diversity and population structure of *Robinia pseudoacacia* from six improved variety bases in China as revealed by simple sequence repeat markers. J For Res 33:611-21. https://doi.org/10.1007/s11676-021-01356-2.

Huang W, Yang Y-J, Hu H, Zhang S-B, Cao K-F. 2016. Evidence for the role of cyclic electron flow in photoprotection for oxygen-evolving complex. J Plant Physiol 194:54-60. https://doi.org/10.1016/j.jplph.2016.02.016.

Kerchev PI, Fenton B, Foyer CH, Hancock RD. 2012. Plant responses to insect herbivory: interactions between photosynthesis, reactive oxygen species and hormonal signaling path ways. Plant Cell Environ 35:441-53. https://doi.org/10.1111/j.1365-3040.2011.02399.x.

Koch KG, Chapman K, Louis J, Heng-Moss T, Sarath G. 2016. Plant tolerance: a unique approach to control hemipteran pests. Front Plant Sci 7:1363. https://doi.org/10.3389/fpls.2016.01363.

Lazzaro L, Mazza G, d'Errico G. 2018. How ecosystems change following invasion by *Robinia pseudoacacia*: Insights from soil chemical properties and soil microbial, nematode, microarthropod and plant communities. Sci Total Environ 622:1509-18. https://doi.org/10.1016/j.scitotenv.2017.10.017.

Lebeda A, Luhova L, Sedlarova M, Jancova D. 2001. The role of enzymes in plant fungal pathogens interactions / Die Rolle der Enzyme in den Beziehungen zwischen Pflanzen und pilzlichen Erregern. J Plant Dis Prot 108(1):89-111.

Le Gall, Behmer ST. 2014. Effects of protein and carbohydrate on an insect herbivore: The vista from a fitness landscape. Integr Comp Biol 54(5). https://doi.org/10.1093/icb/icu102.

Li K, Han X, Ni R. 2021. Impact of *Robinia pseudoacacia* stand conversion on soil bacterial communities composition and soil properties in Mount Tai, China. Forest Ecosyst 8(19):1-23. https://doi.org/10.1186/s40663-021-00296-x.

Liu Z, Mo K, Fei S, Zu Y, Yang L. 2017. Efficient approach for the extraction of proanthocyanidins from *Cinnamomum longepaniculatum* leaves using ultrasonic irradiation and an evaluation of their inhibition activity on digestive enzymes and antioxidant activity in vitro. J Sep Sci 40(15):3100-13 https://doi.org/10.1016/j.arabjc.2021.103328.

Luhová L, Hedererová D, Lebeda A, Peč P. 2002. The influence of *Fusarium solani* on enzyme activity of *Pisum sativum* cultivars / Der Einfluss von *Fusarium solani* auf die Aktivitäten einiger Enzyme in Erbsensorten. Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz. J Plant Dis Prot 109(2):113-28.

Luo Q, Peng M, Lei P et al. 2017. Comparative mitochondrial proteomic, physiological, biochemical and ultrastructural profiling reveal factors underpinning salt tolerance in tetraploid black locust (*Robinia pseudoacacia* L.). BMC Genomics 18:648:3-23. https://10.1186/s12864-017-4038-2.

Mathakutha R, Steyn Ch, le Roux PC et al. 2019. Invasive species differ in key functional traits from native and non-invasive alien plant species. J Veg Sci 30(5):994-1006 https://doi.org/10.1111/jvs.12772.

McHale L, Tan X, Koehl P, Michelmore RW. 2006. Plant NBS-LRR proteins:adaptable guards. Genome Biol 7(4):212. https://doi.org/10.1186/gb-2006-7-4-212.

Mithöfer A, Boland W. 2012. Plant defense against herbivores:chemical aspects. Annu Rev Plant Biol 63:431-50. https://doi.org/10.1146/annurev-arplant-042110-103854.

Nicolescu VN, Rédei K, Mason WL et al.

2020. Ecology, growth and management of black locust (*Robinia pseudoacacia* L.), a non-native species integrated into European forests. J For Res 31:1081-101. https://doi.org/10.1007/s11676-020-01116-8.

Passardi F, Cosio C, Penel C, Dunand C. 2005. Peroxidases have more functions than a Swiss army knife. Plant cell Rep 24(5):255-65. https://doi.org/10.1007/s00299-005-0972-6.

Paterska M, Bandurska H, Wysłouch J, Molińska-Glura M, Moliński K. 2017. Chemical composition of horse-chestnut (Aesculus) leaves and their susceptibility to chestnut leaf miner *Cameraria ohridella* Deschka & Dimić. Acta Physiol Plant 39(105) 1-16. doi.org/10.1007/S11738-017-2404-Y.

Pyšek P, Jarošík V, Hulme Ph et al. 2012. A global assessment of invasive plant impacts on resident species, communities and ecosystems: The interaction of impact measures, invading species' traits and environment. Global Change Biol 18(5):1725-37. https://doi.org/10.1111/j.1365-2486.2011.02636.x.

Ray S, Gaffor I, Acevedo FE et al. 2015. Maize plants recognize herbivore-associated cues from caterpillar frass. J Chem Ecol 41:781-92. https://doi.org/10.1007/s10886-015-0619-1.

Seliutina OV, Shupranova LV, Holoborodko KK et al. 2020. Effect of *Cameraria ohridella* on accumulation of proteins, peroxidase activity and composition in *Aesculus hippocastanum* leaves. Regul Mech Biosyst 11(2):299-304. https://doi.org/10.15421/022045.

Shupranova LV, Holoborodko KK, Seliutina OV, Pakhomov OY. 2019. 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. Biosyst Divers 27(3):238-43. https://doi.org/10.15421/011933.

Shupranova L, Holoborodko K, Loza I, Zhukov O, Pakhomov O. 2022. Assessment of *Parectopa robiniella* Clemens (Lepidoptera: Gracillariidae) effect on biochemical parameters of *Robinia pseudoacacia* under conditions of an industrial city in steppe Ukraine. Ekológia (Bratislava) 41(4):340-50. https://doi.org/10.2478/eko-2022-0035.

Smith CM, Clement SL. 2012. Molecular bases of plant resistance to arthropods. Annu Rev Entomol 57:309-28. https://doi.org/10.1146/annurev-ento-120710-100642.

Środek D, Rahmonov O. 2022. The Properties of Black′ Locust *Robinia pseudoacacia* L. to Selectively Accumulate Chemical Elements from Soils of Ecologically Transformed Areas. Forests 13:7. https://doi.org/10.3390/f13010007.

Temple M, Perrone G, Dawes I. 2005. Complex cellular responses to reactive oxygen species. Trends Cell Biol 15(6):319-26. https://doi.org/10.1016/j.tcb.2005.04.003.

Turfan N, Alay M, Sariyildiz T. 2018. Effect of tree age on chemical compounds of ancient Anatolian black pine (*Pinus nigra* subsp. *pallasiana*) needles in Northwest Turkey. Forest - Biogeosci For 11(3):406-10. https://doi.org/10.3832/ifor2665-011.

Tyśkiewicz K, Konkol M, Kowalski R et al. 2019. Characterization of bioactive compounds in the biomass of black locust, poplar and willow. Trees 33:1235-63. https://doi.org/10.1007/s00468-019-01837-2.

Tytar V, Nekrasova O, Marushchak O. 2022. The spread of the invasive locust digitate leafminer *Parectopa robiniella* Clemens, 1863 (Lepidoptera: Gracillariidae) in Europe, with special reference to Ukraine. Diversity 14(605):1-13. https://doi.org/10.3390/d14080605.

Tzvetkova NP, Petkova K. 2015. Bioaccumulation of heavy metals by the leaves of *Robinia pseudoacacia* as a bioindicator tree in industrial zones. J Environ Biol 36(1):59-63.

Usha RP, Jyothsna Y. 2010. Biochemical and enzymatic changes in rice as a mechanism of defense. Acta Physiol Plant 32:695-701. http://dx.doi.org/10.1007/s11738-009-0449-2.

Vítková M, Sádlo J, Roleček J, Petřík P, Sitzia T, Müllerová J, Pyšek P. 2020. *Robinia pseudoacacia*-dominated vegetation types of Southern Europe: Species composition, history, distribution and management. Sci Total Environ 707:134857. https://doi.org/10.1016/j.scitotenv.2019.134857.

War AR, Paulraj MG, Ahmad T. 2012. Mechanisms of plant defense against insect herbivores. Plant Signal Behavor 7 (10):1306-20. https://doi.org/10.4161/psb.21663.

Wielkopolan B, Obrępalska-Stęplowska A. 2016. Three-way interaction among plants, bacteria, and coleopteran insect. Planta 244(2):313-32. https://doi.org/10.1007/s00425-016-2543-1.

Yang H, Zhou C, Wu F, Cheng J. 2010. Effect of nitric oxide on browning and lignification of peeled bamboo shoots. Postharvest Biol Technol 57(1):72-6. https://doi.org/10.1016/j.postharvbio.2010.02.004.

Zhao H, Sun X, Xue M, Zhang X, Li Q. 2016. Antioxidant enzyme responses induced by whiteflies in tobacco plants in defense against aphids: Catalase may play a dominant role. PLoS ONE 11(10):e0165454. https://doi.org/10.1371/journal.pone.0165454.

Zhu F, Poelman EH, Dicke M. 2014. Insect herbivore-associated organisms affect plant responses to herbivory. New Phytol 204(2):315-21. https://doi.org/10.1111/nph.12886.