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Article

Susceptibility of Different *Aesculus* Species to the Horse Chestnut Leaf Miner Moth: Chemical Composition and Morphological Features of Leaves

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Abstract: The susceptibility of seven species of chestnuts to the horse chestnut leaf miner was studied in the arboretum of the Main Botanical Garden of the Russian Academy of Sciences (MBG RAS), taking into account their interspecific characteristics. Using pheromone delta traps, the highest number of *Cameraria ohridella* moths was shown for *Aesculus hippocastanum* and the lowest for *Aesculus chinensis*. A number of anatomical parameters of leaves were investigated, such as the thickness of the epidermal cell wall and the thickness of the palisade and spongy parenchyma layers. As a result, it was shown that the most infected chestnut species had a greater thickness of the nutritious parenchyma tissue. No dependence was found between the degree of susceptibility to the horse chestnut leaf miner and such indicators as the content of chlorophyll a + b and carotenoids in the leaves of seven species of chestnuts. Nevertheless, resistance of different species of the genus *Aesculus* to *Cameraria ohridella* under increased tannin content in leaves has been shown. Evaluation of phenolic compounds and flavonoids has not established their reliable role as repellents. The high levels of carbohydrates found during the study contributed to increased susceptibility to the horse chestnut leaf miner.

Keywords: *Cameraria ohridella*; *Aesculus* spp.; infestation patterns; horse chestnut leaf miner; leaves; organic component analysis

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1. Introduction

The *Aesculus* L. is a genus of the family *Hippocastanaceae*, consisting of 12 species dispersed throughout the northern hemisphere and divided into five sections: *Aesculus* L., *Calothyrsus* (Spach) K. Koch, *Pavia* (Mill) Persoon, *Macrothyrsus* (Spach) K. Koch, and *Parryanae* Wiggins [1]. Some species of *Aesculus* are used in Europe as ornamental trees. In particular, horse chestnut (*Aesculus hippocastanum* L.) has been cultivated since 1576 [2]. Moreover, *A. hippocastanum* is the only species of the genus *Aesculus* of European (Balkan) origin and the main host plant of the horse chestnut leaf miner *Cameraria ohridella*, which was first recorded in Macedonia in the 1970s and described as a new species in 1986 [3,4]. In 1989, *C. ohridella* was revealed in Austria, and since then the leaf miner has spread rapidly and penetrated into most European countries. In Russia, *C. ohridella* was first registered in

2003 in the Kaliningrad region [5,6]. Currently, the species are found in 13 central regions of the European part of Russia, as well as in the south of Russia, in particular, in the Krasnodar region, where it produces impressive outbreaks in resort areas [6,7]. In the north of the European part of Russia, *C. ohridella* was noted in St. Petersburg in 2013. The Volga region is known as the extreme eastern limit of detection of *C. ohridella* [7]. The species was found here in 2018, indicating its expansion from the western regions of the European part of Russia.

A study of horse chestnut leaf miner populations in European Russia by Kirichenko et al. [6] identified only two haplotypes (A and B) of *C. ohridella* out of 44 known leaf miner haplotypes in the natural range of *A. hippocastanum* [6]. At the same time, haplotype A was absolutely dominant and was present in all studied populations, while haplotype B was rare.

Early observations in European countries confirm the ability of *C. ohridella* to switch to other chestnut species in areas with heavily infested horse chestnut trees [8,9]. Several previous studies have reported that different *Aesculus* species differ in their susceptibility to *C. ohridella* [10,11]. Thus, for 11 species from the chestnut collection at the Royal Botanic Gardens, Kew (RGB), D'Costa et al. [1]. assessed the susceptibility of individual chestnut species based on infestation levels, leaf miner egg density, larval survival, and consequent leaf damage caused by the leaf miners [1]. They found that *C. ohridella* laid eggs on all studied species, but the highest egg density was recorded on *A. hippocastanum* and *A. turbinata.*

Certainly, botanical gardens provide an ideal opportunity to compare the ability of *C. ohridella* to grow on different chestnut species growing under similar environmental conditions. Thus, phytophage mines were found on A. hippocastanum trees in the arboretum of the Main Botanical Garden of the Russian Academy of Sciences (MBG RAS) and were first discovered in 2005, so several generations of *C. ohridella* had the opportunity to interact with a variety of species in the collection of representatives of the genus Aesculus in the MBG RAS [5]. This fact made it possible to study in more detail the biology of the development of the horse chestnut leaf miner, taking into account the interspecific characteristics of individual species of chestnuts. It is known that each type of chestnut has its own composition of multifunctional phenolic compounds, which are one of the main factors protecting the plant from pathogens and arthropod pests [12,13]. In particular, hydrolyzable tannins act as inhibitors and thus reduce the digestibility of plant tissues, especially deciduous trees [14].

In addition, an important role is played by the anatomical features of the chestnut leaf, such as the presence of cuticles and hairs and the thickness of the outer cell wall. Since the location of stomata in the abaxial epidermis, one layer of palisade parenchyma, and the larger cell size of the adaxial epidermis compared to the abaxial epidermis may be a physiological and mechanical barrier to the nutrition of *C. ohridella* larvae [15].

Thus, the size of the pest population depends on a number of factors. For example, Sefrova and Lastuvka [16] evaluated the distribution of *C. ohridella* in Europe over a 10-year period and found that the transfer of the pest by air currents is the most important factor in the spread of this species [16]. Female *C. ohridella* also attracts males for mating using a sex pheromone, the main component of which has recently been identified [17,18]. Its synthetic analog is successfully used in monitoring systems with high efficiency. Among the known trap systems, delta traps are characterized by low cost and ease of operation, but their sticky surface may lose its stickiness over time [19]. The aim of our work was to determine the morphological and biochemical characteristics of leaves of representatives of the genus *Aesculus* L. in relation to the damage of the horse chestnut leaf miner.

2. Materials and Methods

2.1. Place of Research and Plant Material

The object of study was a collection of *Aesculus* species on the territory of the Main Botanical Garden of the Russian Academy of Sciences in Moscow (55.838° N, 37.588° E)

(Table 1, Supplementary Materials). Two to three trees of each of the seven species infected with the horse chestnut leaf miner were selected for the study. In 2023 (first year) and 2024 (second year), phytomonitoring of plants was carried out, including an analysis of the number of the first generation (from mid-June to early July) of the horse chestnut leaf miner. Pheromone traps were used, as well as a cytological and biochemical analysis of leaves from adult trees with signs of damage by the moth during the period of active feeding.

Table 1. Composition of various *Aesculus* species in MBG RAS [20–22].

Section	Species	GPS of the Location of the Trees in the Chestnut Collection MBG RAS	The Number of Trees in the Collection	Accession Number, MB RAS	Description
Aesculus L.	*A. hippocastanum* L.	1a-55.845042; 37.599364 1b-55.845123; 37.599442 1c- 54.845057; 37.599240	17 pieces	1950-149710; 1953-149713; 1954-3327; 1959-49734	Endemic to the Balkan Peninsula, it can be found in Bulgaria, Greece, and Albania. Widely cultivated in Europe and North America. Approximately 25–30 m in height, it has dense white flowers and 5 or 7 cuneate-obovate leaflets. Since 1941, 22 accessions were grown in MBG RAS from seeds obtained from various botanical gardens.
Pavia (Mill.) Persoon	*A. glabra* Willd	2a-55.845395; 37.599834 2b-55.845491; 37.599748 2c-55.845473; 37.599990	6 pieces	1950-3448; 1954-3448/45; 1965-3448/65; 1965-149712	10–30 m tall, can be found in Pennsylvania, Iowa, Arkansas, Tennessee, and Alabama. It has yellow flowers. Approximately 5–7 leaflets, oblong-obovate or elliptic-obovate. In dendroculture since 1809, it is widespread in botanical gardens of Europe, Central Asia, and North America. Three accessions were grown from seeds obtained from botanical gardens; there are also plants of the MBS reproduction.
	A. flava Aiton	3a-55.844992; 37.599775 3b-55.844942; 37.599630 3c-55.844981; 37.599931	5 pieces	1953-4182; 1961-95638	Distributed in North America, it is 20–30 m tall and has yellow flowers. Leaves have 5 or 7 leaflets. Three accessions were grown from seeds obtained from different botanical gardens, but there were also plants of MBS reproduction.
	A. pavia L.	4a-55.845072; 37.600070 4b-55.844966; 37.600172	2 pieces	1961-95641; 1965-31217	North American species up to 10 m tall have red flowers. Leaves have 5 or 7 leaflets, oblong, obovate, and narrowly elliptic. Since 1950, one accession has been grown in the MBG from seeds obtained from the Trostyanets Arboretum (Ukraine).

Table 1. *Cont.*

Section	Species	GPS of the Location of the Trees in the Chestnut Collection MBG RAS	The Number of Trees in the Collection	Accession Number, MB RAS	Description
	A. × *carnea* Hayne	5a-55.845253; 37.599973 5b-55.845250; 37.6000150	3 pieces	1960-86340 1964-87325	Tree up to 15 (25) m tall. The hybrid was obtained in culture in 1818. It is found quite often in the south of Europe and North America. In MBS since 1989. Three accessions were grown from seeds received from Holland.
Macrothyrsus (Spach) K. Koch	*A. parviflora* Walter	6a-55.845078; 37.600515 6b-55.845156; 37.600623	2 pieces	No data	North American species, up to 5 m tall. Leaves have 5 or 7 leaflets; elliptic to oblong-obovate. It has white flowers.
Calothyrsus (Spach) K. Koch	*A. chinensis* Bunge	7a-55.845250; 37.600451 7b-55.845340; 37.600585	2 pieces	No data	Distributed in China, up to 25 m tall and has white flowers. Leaves have 5–7 leaflets, oblong-lanceolate or oblongoblanceolate

2.2. Estimation of Horse Chestnut Miner Abundance Using Pheromone Traps

Delta sticky pheromone traps with dispensers impregnated with the synthesized sex pheromone of female moths (Pheromon, Russia) were hung on horse chestnut trees in different parts of the crown. The first generation of adults in the chestnut collection was counted from mid-June to early July. Traps in three biological replicates on two to three trees were attached to horizontal branches of the outer part of the chestnut crown at a height of 1.5–2 m from the ground [5]. Sticky plates in the traps were extracted once.

In this study, only the abundance of the first generation of males of the horse chestnut leaf miner was investigated in order to avoid the influence of intraspecific competition between the first and second generations of males of the horse chestnut leaf miner for food.

2.3. Scanning Electron Microscopy

Fragments of the leaf middle part with a size of 2–3 mm were fixed during the day at +4 °C in a 2.5% solution of glutaraldehyde (Merck, Germany) prepared in 0.1 M phosphate buffer (pH is 7.2) with the addition of 1.5% sucrose. Then, the samples were dehydrated at +4 °C for 30 min in each alcohol solution with successively increasing concentrations: 30%, 50%, 70%, 96%, and in three changes of absolute ethanol. After that, the samples were transferred to liquid CO_2 under pressure in the device for drying at the "critical point" and slowly heated under pressure. When the pressure and temperature together passed the so-called "critical point" (31 °C and 74 bar), the pressure was reduced, thus drying the samples without any damage. Next, a thin (from 1 nm to more) metal layer was deposited onto the samples to enhance conductivity and add mechanical strength to the sample (sputtering unit SPI supplies, SPI, Santa-Clara, CA, USA). The photographs were obtained using a JSM-6380LA scanning electron microscope (JEOL Ltd., Tokyo, Japan) [5].

2.4. Determination of Dry Matter of Leaves

The dry matter content in the leaves of species chestnuts was determined according to the generally accepted method of the National Standard of the Russian Federation (GOST 31640-2012) [23].

2.5. Determination of Leaf Pigment Content

The content of chlorophyll a, chlorophyll b, and the sum of carotenoids in fresh chestnut leaves were determined spectrophotometrically using a Spekol 1300 spectrophotometer (Analitik Jena AG, Jena, Germany) due to Lichtenthaler method [24].

2.6. Determination of Tannin Content

The tannin content in terms of tannin was determined according to the method described in OFS. 1.5.3.0008.18 GF XIV, "Determination of the content of tannins in medicinal plant materials and medicinal plant preparations", by the permanganatometry method [25].

2.7. Determination of Phenolic Compounds and Flavonoids

Total polyphenol content was measured spectrophotometrically on a Spekol 1300 spectrophotometer (Analitik Jena AG, Jena, Germany) using the Folin–Ciocalteu reagent according to the method described in detail in [26]. Gallic acid (25–300 mg/L; R2 = 0.998) was used as a standard. The results were expressed as mg/g gallic acid equivalent DW (dry weight) [26].

Total flavonoid content was determined using a modified method described in [27]. A 1 mL aliquot of each sample was mixed with 2 mL of a 2% (*w*/*v*) ethanol solution of aluminum chloride, 0.5 mL of 1 M hydrochloric acid, and 6.5 mL of ethanol (96%). After 20 and 40 min in the dark, the absorbance at 415 nm was measured using a Spekol 1300 spectrophotometer (Analitik Jena AG, Jena, Germany). Quercetin (1–400 mg/L; R2 = 0.9977) was used as standard. The results were expressed as mg/g quercetin equivalent on a DW (dry weight) basis [27].

2.8. Determination of the Composition of Phenolic Compounds in Chestnut Leaves

From leaves of seven chestnut species on which damage from *C. ohridella* was recorded, averaged samples were formed. Leaf powder samples (1 g) were extracted with methanol (20 mL). Extraction was carried out for 20 min with sonication and occasional shaking. Then, the suspension was centrifuged at 15,000× *g* for 15 min, and the supernatant was filtered through a 0.25 µm membrane and evaporated to dryness in a helium stream. The identification and content of secondary metabolites in individual extracts were determined by chromato-mass spectrometry (GC-MS) on a JMS-Q1050GC GC-MS chromatograph (JEOL Ltd., Tokyo, Japan). The conditions for determining the composition of phenolic compounds are described in more detail in a previously published paper [5].

2.9. Statistical Analysis

The thickness of the leaf blade was calculated using the Image J program with an accuracy of 0.1 µm. At least 300 cells of the above tissues from three independent leaves were analyzed for each experimental treatment. To compare the arithmetic means, an ANOVA was used with the Bonferroni correction. The measurements were performed in the Statistica v. 12.0 PL (StatSoft, Tulsa, OK, USA) program. The abundance of chestnut leaf miners was measured in 2-fold biological and 2-fold analytical replicates. All measurements and determinations of biochemical parameters in the leaves of species chestnuts were performed in 2-fold biological and 3-fold analytical replicates, and average values were used in the calculations. The thickness of the leaf blade was calculated using the Image J program with an accuracy of 0.1 µm. At least 300 cells of the above tissues from three independent leaves were analyzed for each experimental treatment. To compare the arithmetic means, an ANOVA was used with the Bonferroni correction. The measurements were performed in the Statistica v. 12.0 PL (StatSoft, Tulsa, OK, USA) program.

3. Results

The study revealed differences in the abundance of horse chestnut leaf miner on different chestnut species. In the first year, the highest number of *C. ohridella* was found on *A. hippocastanum* plants (380 moths on average in a trap), and the lowest number of moths

(11 males on average in a trap) was shown in the *A. chinensis* species, with a difference 12 times smaller than in *A. hippocastanum*. Next in the abundance of the horse chestnut leaf miner were the species *A. glabra* and *A. flava* (187 and 173 males, respectively), which did not significantly differ from each other in the number of moths in the trap. Even less populated by the horse chestnut leaf miner were the species *A. pavia* (65 males), *A.* × *carnea* (89 males) and *A. parviflora* (63 males), between which no differences were found either. In the second year, the trend in the number of the first generation of males generally remained. However, the number of males in traps for some species was several times greater than in the first year. Thus, this parameter was 1.6 times greater for *A. flava*, 2.5 times greater for *A. pavia*, 2.1 times greater for *A.* × *carnea*, 3.1 times greater for *A. parviflora*, and 7 times greater for *A. chinensis* (Figure 1).

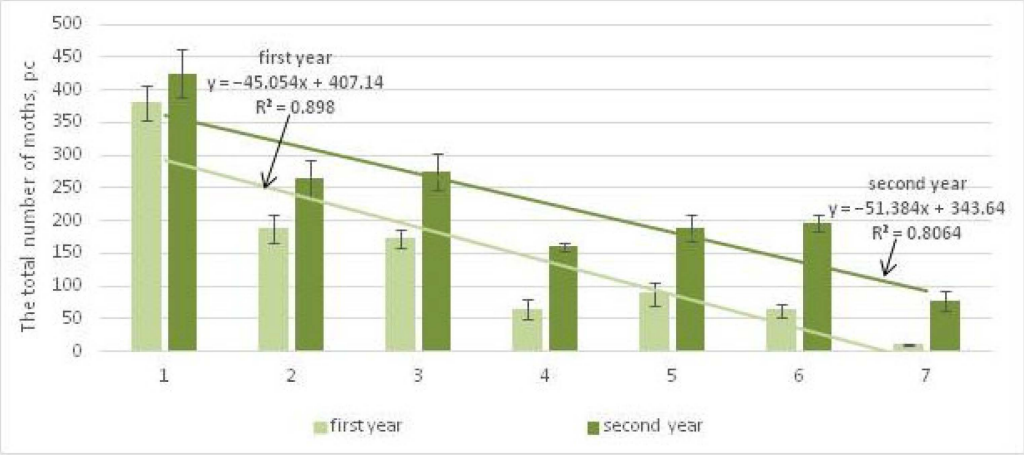

Figure 1. Total number of first-generation males *C. ohridella* in 2023 (first year) and 2024 (second year). Standard deviations are given as the margin of error. 1—*A. hippocastanum*; 2—*A. glabra*; 3—*A. flava*; 4—*A. pavia*; 5—*A.* × *carnea*; 6—*A. parviflora*; 7—*A. chinensis*.

The characteristic features of the leaves of different species of horse chestnut were studied using morphological assessment of the leaf cross-section (Figure 2). This analysis was carried out only in the first year of observation. These *Aesculus* species showed a structure typical for mesomorphic leaves, caused by the climatic conditions of the environment. The leaves, regardless of the species, have thin outer cell walls of the epidermis, one palisade, and spongy parenchyma.

The thickness of the cell wall of the upper epidermis was significantly higher in *A. glabra* and *A. flava* plants (2.7 and 3 μm, respectively). In *A. chinensis* and *A. parviflora*, the cell wall thickness was the smallest of all the studied chestnut species in the collection and amounted to 1.6 μm (Figure 3a). Thus, in *A. chinensis* plants, this indicator was 2 times smaller than in *A. flava*.

A similar trend was characteristic of the thickness of the palisade parenchyma of chestnut leaves (Figure 3b). In the species *A. glabra* and *A. flava*, this indicator was the largest and did not statistically differ from each other (42.8 and 46.3 μm, respectively). The palisade parenchyma thickness was smaller in *A. hippocastanum* (37.4 μm) and *A.* × *carnea* (35.8 μm), and the smallest value was recorded in *A. pavia* (23.9 μm) and *A. chinensis* (23.2 μm).

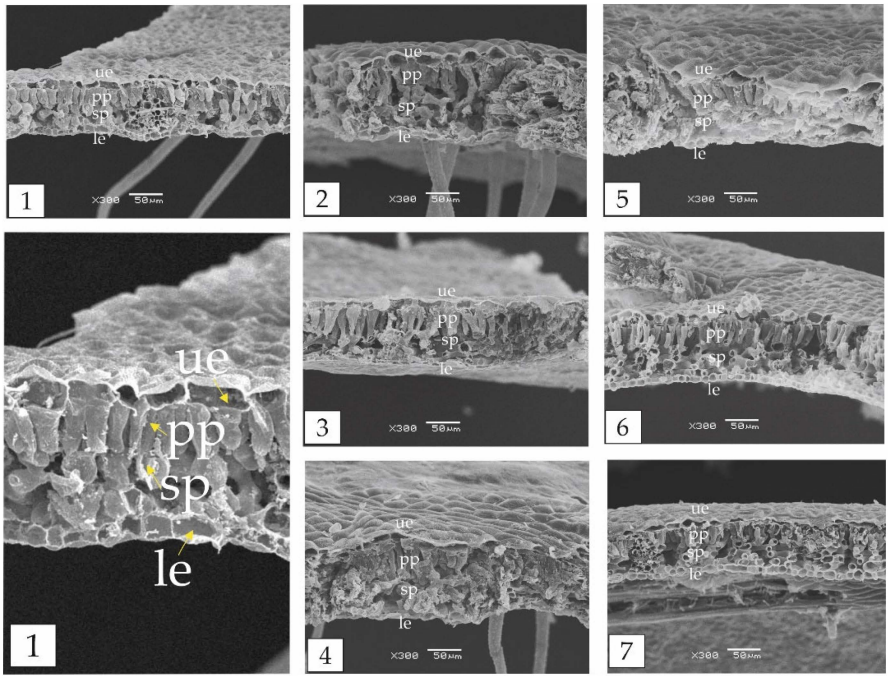

Figure 2. Cross sections of *C. ohridella*-infested *Aesculus* sp. using scanning electron microscopy. Abbreviations: ue—upper epidermis, le—lower epidermis, pp—palisade parenchyma, sp—spongy parenchyma. 1—*A. hippocastanum*; 2—*A. glabra*; 3—*A. flava*; 4—*A. pavia*; 5—*A.* × *carnea*; 6—*A. parviflora*; 7—*A. chinensis*.

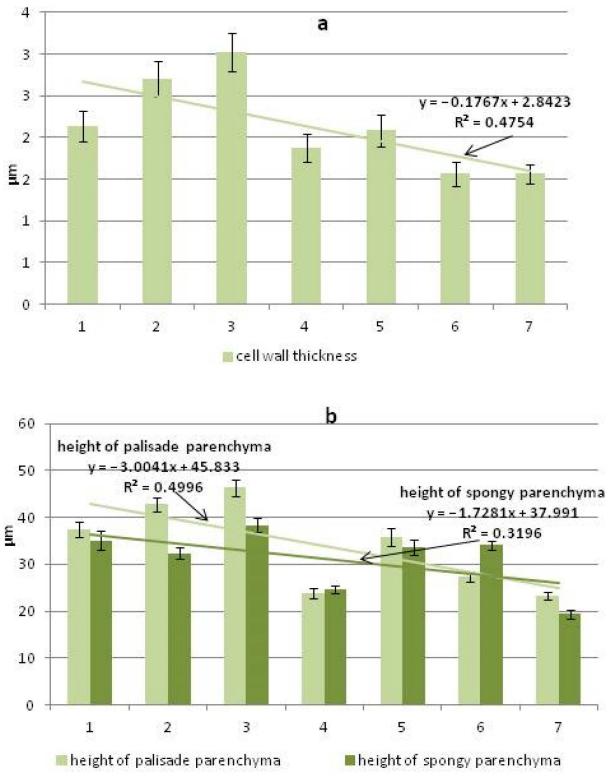

Figure 3. Morphometric characteristics of anatomical differences in leaf blade mesophyll cuts of horse chestnut leaf. Cross sections of *Cameraria ohridella*-infested *Aesculus* leaves. Standard deviations are given as the margin of error. Abbreviations: (**a**)—thickness of the cell wall of upper epidermis; (**b**)—height of parenchyma. 1—*A. hippocastanum*; 2—*A. glabra*; 3—*A. flava*.; 4—*A. pavia*; 5—*A.* × *carnea*; 6—*A. parviflora*; 7—*A. chinensis*.

A. flava also had the greatest thickness of spongy parenchyma, equal to that of *A. hippocastanum*, which were not statistically different from each other (38.3 and 35.1 μm, respectively). The thickness of spongy parenchyma, as in the case of columnar parenchyma, was the smallest in *A. pavia* (24.7 μm) and *A. chinensis* (19.4 μm).

The pigment composition is the main indicator characterizing the functioning of the photosynthetic apparatus. In turn, photosynthesis is the most important process that ensures the resistance of plants to biotic and abiotic stress. During the first year of observation, it was revealed that in the leaves of most of the analyzed chestnuts, the sum of chlorophylls C_{chla} + C_{chlb} was 0.37–0.45 mg/g DW, and only in the leaves of *A. parviflora* was this indicator significantly higher—0.76 mg/g DW. No significant differences in chlorophyll content were recorded between species (R^2 = 0.15) (Figure 4a). In this case, chlorophyll b in four species (*A. hippocastanum*, *A. pavia*, *A. flava*, and *A.* × *carnea*) was 31–38% of C_{chla} + C_{chlb}, and, consequently, the ratio of chlorophyll a to chlorophyll b was significantly higher. This indicated a rearrangement of the photosynthetic apparatus in the leaves of these species towards an increase in the proportion of Photosystem I. In the leaves of *A. glabra*, *A. parviflora*, and *A. chinensis*, chlorophyll b was 54–56% of C_{chla} + C*chlb*. The high level of chlorophyll b resulted in lower C_{chla}/C_{chlb} values and may indicate that the relative amount of Photosystem II increased in the structure of the chlorophyll complex of the leaves of these species. In the second year of observation, the total chlorophyll content increased in six species of chestnuts (0.42–0.60 mg/g DW) and decreased slightly in the leaves of *A. parviflora*—0.68 mg/g DW. No significant differences in chlorophyll content between species were also recorded (R^2 = 0.35) (Figure 4b). Only two chestnut species (*A. hippocastanum* and *A.* × *carnea*) had a high chlorophyll a: chlorophyll b ratio—3.46 and 3.00, respectively. For the remaining species, the C_{chla}/C_{chlb} ratio ranged from 2.15 to 2.59. No significant differences were revealed between species for this indicator in either the first or second year of observation (R^2 = 0.06 and R^2 = 0.43, respectively).

The ratio of chlorophyll a: chlorophyll b to the sum of carotenoids (a + b)/(x + c) is an indicator of the normal functioning of the photosynthetic apparatus of plants or the so-called greenness of plant leaves. In the first year of observation, the ratio (a + b)/(x + c) in the leaves of 4 species of chestnuts fluctuated from 4.2 to 5, which indicates a high degree of illumination of the tree crowns (Figure 4c). Higher values of this indicator recorded in the leaves of *A. parviflora* and *A. chinensis*—6.2 and 6.9, respectively, indicated that these plants grow in more shaded conditions. And only in the most affected species (*A. hippocastanum*) this indicator was 3.5, which is an indicator of aging, stress, and damage to the plant and its photosynthetic apparatus. This can manifest in a faster breakdown of chlorophylls than carotenoids. In the second year of observation, a generally similar picture was recorded. However, in the leaves of *A. hippocastanum*, this indicator was higher—4.02, which indicates a more favorable state of the photosynthetic apparatus of the leaves of this species.

In the first year of observation, the minimum amount of tannins (2.09% DW) was recorded in the leaves of *A. hippocastanum*, the species most susceptible to *C. ohridella* (Figure 5a). In two other species (*A. glabra*, *A. flava*) with a high degree of susceptibility to orchid leaf miner, the tannin content was 1.4 times higher. In three species partially infected with *C. ohridella*, tannin content also increased compared to *A. hippocastanum*. And the maximum amount of tannins (4.77% DW) in the leaves of the horse chestnut leaf miner-resistant *A. chinensis* was revealed. It is possible that the high concentrations of tannins in the leaves explain the greater resistance of *A. chinensis* to *C. ohridella* pests. It should be noted that the content of these compounds significantly decreased with an increase in the degree of susceptibility of plants to the horse chestnut leaf miner—R2 = 0.75. In the second year of observation, the tannin content in the leaves of 5 chestnut species was almost the same—3.08–3.52% DW. And only in two species, *A. parviflora* and *A. chinensis*, it was slightly higher—4.02 and 5.12% DW, respectively. No significant differences were found between species in this indicator.

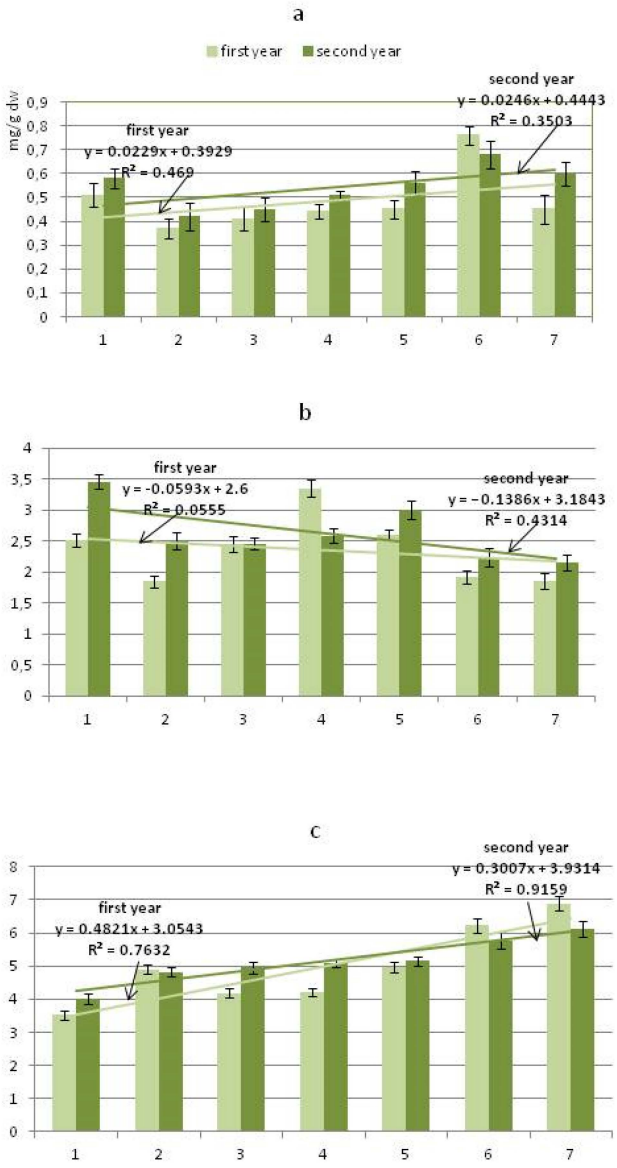

Figure 4. Content of total chlorophylls (**a**), ratio C_{chla}/C_{chlb} (**b**) and ratio of chlorophyll a: chlorophyll b to total carotenoids (**c**) in different species of chestnut. 1—*A. hippocastanum*; 2—*A. glabra*; 3—*A. flava*; 4—*A. pavia*; 5—*A.* × *carnea*; *6*—*A. parviflora*; 7—*A. chinensis*.

The total content of phenolic compounds in the leaves of 7 species of chestnuts in the first year of observation varied significantly in plants with different degrees of susceptibility to the horse chestnut leaf miner (Figure 5b). The phenolic content in A. hippocastanum leaves was 5.35 mg/g DW. In contrast, the concentration of polyphenolic compounds in uninfected leaves of *A. chinensis* was significantly lower and reached 1.97 mg/g DW. In the second year of observation, the total phenolic compounds fluctuated less significantly. Thus, in three species susceptible to the horse chestnut leaf miner, it varied from 3.8 to 3.3 mg/g DW, while in the remaining species it varied from 2.9 to 2.1 mg/g DW. Reliable differences were noted between the species for this indicator—$R^2 = 0.85$ and $R^2 = 0.80$ in the first and second years of observation, respectively.

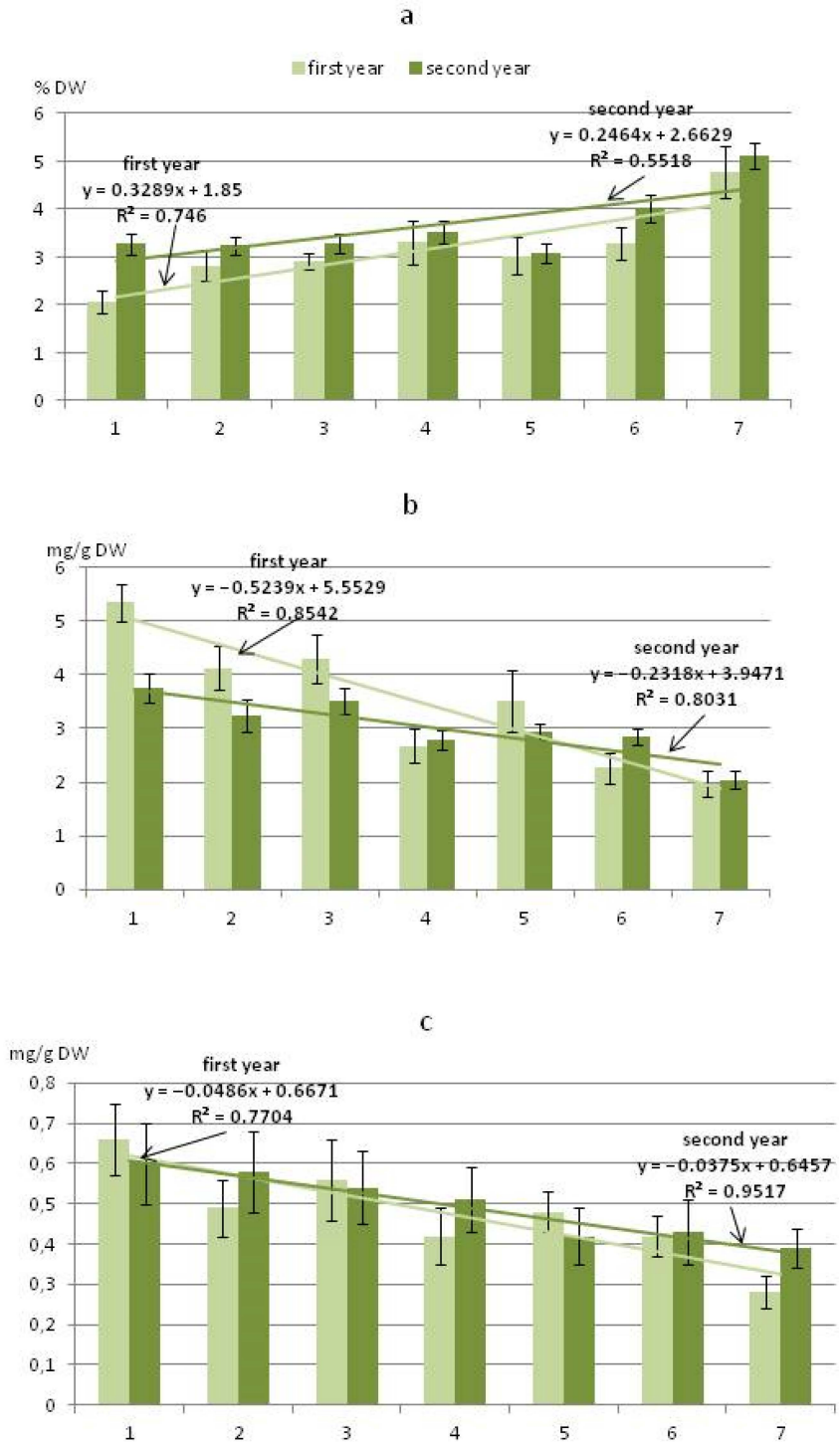

Figure 5. The amount of tannins (**a**), phenolic compounds (**b**), and flavonoids (**c**) in different chestnut species. 1—*A. hippocastanum*; 2—*A. glabra*; 3—*A. flava*; 4—*A. pavia*; 5—*A.* × *carnea*; 6—*A. parviflora*; 7—*A. chinensis*.

The total content of quercetin series flavonoids in the leaves of 7 species of chestnuts also differed significantly in plants with different degrees of susceptibility to the horse chestnut leaf miner in the first year of observation (Figure 5c). *A. hippocastanum* leaves contained 0.66 mg/g DW quercetin, which was 2.4 times higher than that of *A. chinensis*. In the second year of observation, the difference in the total flavonoids in the leaves of these species, although preserved, was not so significant—1.5 times. Reliable differences

between species for this indicator were also recorded—R^2 = 0.77 and R^2 = 0.95 in the first and second years of observation, respectively.

In general, it should be noted that in the leaves of chestnut species affected by the attack of the horse chestnut leaf miner, the concentration of polyphenolic compounds and quercetin flavonoids was higher than in species less infected with *C. ohridella*.

Analysis of the main substances contained in the methanol extract prepared from the leaves of seven species of chestnuts was carried out only in the first year of observation. Thirty-five metabolites were identified by gas chromatography-mass spectrometric (GC-MS) analytic technique. Their peak heights were at least 0.1% of the instrument scale (Table 2).

Table 2. Composition of organic components in the leaves of different chestnut species. 1—*A. hippocastanum*; 2—*A. glabra*; 3—*A. flava*; 4—*A. pavia*; 5—*A.* × *carnea*; 6—*A. parviflora*; 7—*A. chinensis*. (% mass. of the extract).

	1	2	3	4	5	6	7
				Polyphenols			
Glycerol	1.91	3.92	12.03	5.17	4.45	14.49	0.01
1,2,2-3-Butanetriol	0.27	0.62	0.01	0.79	0.01	0.01	7.80
L-(-)-Arabitol	1.87	4.38	8.81	0.01	2.6	3.41	0.64
D-Fucitol	0.89	0.01	0.01	6.35	0.41	2.30	4.77
D-Mannotol	0.45	0.52	0.01	0.01	0.01	0.01	2.02
1,5-Angidroglucitol	11.04	11.67	8.75	4.62	2.13	0.01	5.35
Scillo-Inositol	1.75	1.72	0.00	0.20	0.95	0.01	0.01
Galactinol	14.19	5.96	1.91	0.60	2.84	0.60	0.44
D-Glucitol	0.95	0.74	2.35	2.38	0.01	0.01	0.01
Maltitol	2.85	2.05	0.57	0.01	0.01	0.01	0.01
Adonitol	0.01	0.01	0.01	0.01	14,11	0.01	0.01
				Organic acid			
Glucopyranuronic acid	0.38	3.97	0.01	23.22	4.45	0.01	22.26
Butanedioic acid	4.92	9.91	11.69	12.87	14.98	17.29	13.27
Quininic acid	6.18	3.65	0.78	0.12	7.47	22.50	4.96
Gluonic acid	4.09	4.67	1.30	5.07	6.63	1.70	5.10
Gallic acid	0.54	0.84	0.29	0.01	0.01	0.01	6.37
Ribonic acid	0.25	0.01	1.94	0.01	1.27	0.01	0.01
D-(+)-Galacturonic acid	0.45	2.29	10.35	0.01	0.01	1.05	5.64
				Sugar derivatives			
D-erythro-2-pentulose	0.62	22.23	0.59	1.11	1.62	8.54	7.42
Methyl-a-D-glucofuranoside	0.75	1.42	3.37	0.28	6.87	13.07	7.29
D-Psicofuranose	9.30	3.72	6.43	0.01	0.82	0.25	3.37
D-(-)-Tagatofuranose	1.45	0.02	13.58	24.49	2.84	3.01	4.05
DL-Arabinofuranoside	3.05	0.01	0.01	4.33	4.86	2.97	0.42
Methyl galactoside	6.04	0.01	4.87	1.33	3.38	1.47	0.84

Table 2. *Cont.*

	1	2	3	4	5	6	7
				Polyphenols			
b-D-(+)-Talophyranose	2.15	0.01	2.99	0.84	0.22	0.05	0.01
Talofuranose	1.57	1.57	0.61	0.09	1.03	0.07	1.11
Deoxyglucose	0.60	13.92	0.78	0.12	2.25	0.10	0.09
a-D-Ribofuranose	0.25	0.99	0.01	0.05	0.77	0.05	0.01
Glucosylspingosine	6.55	0.01	0.01	0.01	1.75	3.21	1.15
D-Turanose	2.98	0.06	2.75	1.11	1.28	0.22	0.20
Methyl-a-N-acetyl-D-galactoside	4.89	0.01	3.18	0.01	0.64	0.01	0.37
D-(-)-Sorbofuranose	1.68	0.01	2.63	0.01	0.75	0.01	0.18
b-Arabinopyranose	2.07	0.01	0.01	0.01	1.63	0.01	0.01
1-c-Octylhexopyranose	7.31	1.01	0.94	1.21	1.16	0.01	0.11
DL-Arabinopyranose	2.78	1.38	0.01	0.01	1.57	0.01	0.63

The metabolites were divided into three main groups: polyphenolic compounds (11 compounds); organic acids (7 compounds) and sugars and their derivatives (17 compounds).

The proportion of polyphenolic compounds ranged from 20.1% (*A. pavia*) to 36.2% (*A. hippocastanum*) of the total composition of leaf metabolites. Three sugar alcohols dominated among the polyphenolic compounds: Glycerol, 1,5-Angidroglucitol, and Galactinol. Among secondary metabolites, the proportion of organic acids varied from 16.8% (*A. hippocastanum*) to 57.6% (*A. chinensis*). Four organic acids dominated: Glucopyranuronic acid, Butanedioic acid, Quininic acid, and Gluonic acid, and in some cases, individual species of this class of compounds accumulated in very large quantities: Glucopyranuronic acid in the leaves of *A. pavia* and *A. chinensis*—up to 23.2 and 22.3%; Quininic acid—up to 22.5% in the leaves of *A. parviflora*. Butanedioic acid was also present in the leaves of all chestnut species, with its content in the leaves of *A. hippocastanum* being 3.5 times lower than in the leaves of *A. parviflora*.

The proportion of carbohydrates in the leaves of 7 species of chestnut ranged from 54.1% to 27.5%. It should be noted that the maximum amount of carbohydrates was found in the leaves of three species of chestnuts—*A. hippocastanum*; *A. glabra*; and *A. flava*—that are most susceptible to the orchid leaf miner— 54.1; 46.3; and 42.7%; respectively. In species with reduced susceptibility to *C. ohridella*, the carbohydrate content in leaves was reduced to 33.0–35.0%. And the minimum amount of carbohydrates was observed in the leaves of *A. chinensis*, a species resistant to the horse chestnut leaf miner. It was 1.97 times less than the content of these compounds in the leaves of *A. hippocastanum*.

4. Discussion

The most damaged and preferred species in the collection can be considered the common horse chestnut. Earlier, during the monitoring of the population of the chestnut leaf miner on the horse chestnut collection, severe damage to *A. hippocastanum* was revealed [5]. This species is the first to be populated by the leaf miner and is most severely damaged during the growing season. Other species, such as *A. glabra and A.* × *carnea*, are either not damaged or have traces of caterpillar penetration into the leaf parenchyma [28]. On species such as *A.* × *hybrida* and *A. pavia*, the caterpillars, although they begin to feed, die at an early stage of development. According to the results of other studies of the damage of various chestnut species, it was found that *A. hippocastanum* and *A. turbinate* are most damaged by *C. ohridella*. Finally, species of the *Pavia* section of North American origin show less susceptibility to the horse chestnut leaf miner. Some individuals in this section were slightly infested or not infested at all [29]. Our study also showed that the highest number

of first-generation males was observed on *A. hippocastanum* plants, while the lowest number of males was shown on *A. chinensis*, *A. pavia*, and *A. parviflora*. The trend was the same in both years, with the only difference being that in some species the horse chestnut leaf miner abundance increased in 2024 compared to 2023 (*A. flava*, *A. pavia*, *A.* × *carnea*, *A. parviflora*, and *A. chinensis*).

This may be related to the phylogeny of the species. Insect activity on nonnative host plants is often found to be related to the phylogeny of the host plant [29]. *C. ohridella* females may be unable to differentiate between some Aesculus species and therefore oviposit on less suitable hosts. Insects often oviposit on plants that are chemically similar to suitable ones [1]. A study by Johne et al. [30] found that infestation of plants with *C. ohridella* resulted in a change in the volatile profile of *A. hippocastanum* leaves. Johne et al. [30] also showed that *C. ohridella* responds to volatiles in *A. hippocastanum* that increase with leaf damage. Finally, another explanation for this result may be that *C. ohridella* has not coevolved with most of the studied species [31].

The cell walls of the epidermis are a mechanical barrier that protects the plant from pathogens and herbivores [32]. The thickness of the outer wall and the presence of cuticles and hairs play an important role. Many authors emphasize that the cuticle layer on the epidermis of plant organs is an important barrier for pathogens and herbivores [33]. Such structural elements of these leaves can serve as a physiological and mechanical barrier for *C. ohridella* larvae feeding in their parenchyma. In the case of horse chestnut leaves, the phytophagous insect can easily overcome the external mechanical barrier consisting of the upper layer of the epidermis and its products [15]. Small cuticular grooves are visible on the cell walls of the adaxial epidermis, but they do not prevent the hatching of *C. ohridella* larvae. Since trichomes on the leaves of *A. hippocastanum* are few and occur only on the veins of the abaxial leaf surface, they probably do not play any role in protection against *C. ohridella* [15]. According to the results of our study, the cell wall thickness was the smallest in the least populated species of the horse chestnut leaf miner *A. chinensis* and *A. parviflora*, while it was significantly thicker in *A. glabra* and *A. flava.* In this regard, it seems that the thickness of the epidermal cell wall is not a serious obstacle to infestation by the pest.

Subsequently, the *C. ohridella* larvae, emerging from the eggs laid on the surface of the chestnut leaf, gnaw through the epidermal layer and reach the palisade parenchyma. In *A. hippocastanum*, the palisade parenchyma layer with thin-walled cells containing a large number of chloroplasts is a valuable food source, easily accessible to herbivores [34]. In our work, it was shown that the chestnut species most populated by the moth (*A. hippocastanum, A. glabra*, and *A. flava*) had the greatest thickness of the palisade and/or spongy parenchyma. At the same time, the species least populated by the pest (*A. pavia* and *A. chinensis*) had a smaller thickness of both types of parenchyma. Thus, the greater susceptibility of some chestnut species to attack than others may be due to the greater thickness (and, consequently, greater food availability for feeding larvae) of the parenchyma.

Photosynthesis is one of the processes most vulnerable to biotic and abiotic stress. Chlorophyll levels change not only during plant vegetation but also as a result of interactions between host plants and insects, such as the horse chestnut leaf miner. Changes in chlorophyll content in plant tissues can be useful for assessing the possibility of using pho-tosynthetic pigments as markers of plant resistance to a particular pathogen. However, according to the available literature, changes in chlorophyll levels do not have a constant pattern and vary depending on the plant species and the type of harmful agent causing stress [35]. Changes in the parameters of chlorophyll fluorescence induction in *A. hippocastanum* leaves depending on the degree of damage to leaf blades by *C. ohridella* caterpillars have been shown in a number of studies [35–37], but it remains unclear whether these changes are reliable. Our results indicate that there is no connection between the chlorophyll content in the leaves of individual chestnut species and their susceptibility to the horse chestnut leaf miner. It is possible that the C_{chla}/C_{chlb} ratio can be used as one of the markers of plant resistance to this phytophage. Low values of this indicator indicate an increase in the structure of the chlorophyll complex of lightharvesting complexes of

photosystem II, the stability of which ensures the adaptive potential of a number of species to the influence of various strains of pathogens. In the first year of observation, the lowest C_{chla}/C_{chlb} value was revealed in two chestnut species resistant to the horse chestnut leaf miner. However, our data from the second year of observation no longer provide such an unambiguous answer to this question. In our opinion, the environmental conditions of the growing season have a greater effect on the state of the photosynthetic apparatus of plants.

The chemical composition of host plants has a significant impact on insect behavior [38]. They affect the olfactory, tactile, and gustatory receptors of herbivores, and their impact on herbivores is often toxic.

In a number of studies, tannins have been described as powerful protective agents that have a significant effect on suppressing the negative activity of arthropod pests, which is consistent with our results. However, at the same time, there is evidence of no difference in the content of tannins in asymptomatic and infected leaves [39].

Among secondary metabolites, plant phenolic compounds represent a very important group of defense compounds that play an important role in resistance to herbivorous insects [14,15,40]. In our study, the concentration of phenols in the leaf blades of species susceptible to the horse chestnut leaf miner was higher, which can be considered a manifestation of chemical defense. Our studies also showed that during two years of observation, the concentration of phenolic compounds in the leaf blades of susceptible species (*A. hippocastanum*, *A. glabra*, and *A. flava*) was higher than in resistant *A. chinensis*. This is generally consistent with the results obtained by D'Costa et al. [41]: higher levels of phenolic compounds were observed in the leaves of species susceptible to *C. ohridella* than in the leaves of resistant species.

Flavonols are compounds with anti-nutritional activity and inhibition of insect development. Increased levels of quercetintype flavonols were observed in *A. hippocastanum* leaves, especially in the first year of observation. An important factor stimulating larval feeding is the abundance of nutrients in the leaves [11]. Insects prefer leaves rich in carbohydrates. The results of our study confirm the important role of carbohydrates in stimulating *C. ohridella* feeding. Susceptible chestnut species, especially *A. hippocastanum*, had significantly higher levels of carbohydrates in the leaves compared to resistant *A. chinensis*. Similar results were obtained by Paterska et al. [38] and in our previous studies [5], but contradict the data of D'Costa et al. [41]: according to PCA, the sugar content of chestnut leaves affects to a lesser extent (PC2 16, 7%), and the main contribution is made by the content of phenolic compounds and amino acids (PC1 49.5%).

The obtained results show that the chemical composition of chestnut leaves is very complex, and the individual biochemical parameters we determined do not fully define the multifaceted interaction of *C. ohridella* with the studied trees of the genus *Aesculus* and do not provide a complete description of the relationships of this pest with different species of the genus *Aesculus*. Probably, at the initial stage of protection, rapid synthesis of phenolic compounds is included, but this turns out to be ineffective, as shown in a number of works and in our research [13,14,39,42]. In addition to the above-described insufficient plant protection strategy associated with the synthesis of phenolic compounds and flavonoids of the quercetin series, high levels of nutrients, in particular carbohydrates, in their leaves play a certain negative role in reducing the resistance of a number of chestnut species to the horse chestnut leaf miner. And only an increased content of tannins presumably explains the greater resistance of individual chestnut species to *C. ohridella*. Therefore, monitoring even tannin content alone can be a reliable indicator/predictor of plant resistance to horse chestnut leaf miners.

5. Conclusions

When taking into account the population size of the first generation of the horse chestnut leaf miner in the territory of the MBG RAS, differences were found between different species of chestnut. The thickness of the cell wall of the upper epidermis does not affect susceptibility to the orchid leaf miner. However, the greater degree of infestation of

Aesculus species by this pest may be due to the greater thickness of the parenchyma, since this provides more food for the feeding larvae.

The content of chlorophyll a + b and carotenoids in the leaves of seven chestnut species did not depend on the degree of their susceptibility to the orchid leaf miner. To a greater extent, changes in the state of the pigment system depended on the environmental conditions of the growing season and specific properties of plants. The resistance of different species of the genus *Aesculus* to *C. ohridella* is largely due to the increased content of tannins in the leaf blade. Phenolic compounds and flavonoids of the quercetin series do not perform a repellent function, and high levels of nutrients, in particular carbohydrates, contribute to increased susceptibility to the horse chestnut leaf miner.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jzbg5040045/s1, Figure S1: Species of chestnuts in the arboretum of the Main Botanical Garden of the Russian Academy of Sciences (MBG RAS).

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