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THE INFLUENCE OF HORSE-CHESTNUT GRAFTING ON CHARACTERISTICS OF RESISTANCE TO CHESTNUT LEAF MINER (CAMERARIA OHRIDELLA DESCHKA & DIMIĆ)

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A number of forms of Aesculus hippocastanum L. are resistant to chestnut leaf miner (CLM) Cameraria ohridella Deschka and Dimić. Here, we studied the influence of grafting scions and rootstock of such forms on persistence of that resistance. Obtaining seedling material by grafting is effective in 60-70 % of cases. Grafted horse-chestnut scions resistant to CLM on two-year old rootstock of vulnerable forms were more susceptible to the pest. Leaves of grafted plants demonstrate signs of deformations and damage associated with CLM for the first two years of vegetation after grafting. CLM infection of plants is accompanied with the increasing concentrations of chlorophylls a and b, and carotenoids. The content of total phenols and catechins also increases in infected leaves. This indicates a restructuring of the secondary metabolism associated with the synthesis of phenolic compounds and terpenoids. Such partial damage together with the increased content of condensed tannins in leaves of CLM-resistant form of horse chestnut confirms the inability of tannins to protect the plant from CLM. It is shown that CLM caterpillars can hydrolyze phenols, in particular proanthocyanidins. Based on our experimental data, phenols are thought to be fermented by intestinal enterobacteria of CLM caterpillars.

Keywords: *Aesculus hippocastanum, scion, rootstock, resistance, Cameraria ohridella, phenols, tannins, carotenoids*

Severe infections of *Aesculus hippocastanum* L. trees with chestnut leaf miner, *Cameraria ohridella* Deschka & Dimić (CLM) adversely impact the overall condition, phytosanitary properties and ornamental quality of horse chestnuts [1, 19].

CLM mostly infects conker trees (*Ae. hippocastanum*) and Japanese horse chestnuts (*Aesculus turbinata* Blume), and is significantly rare on trees of red (*Aesculus x carnea* Zeyh.) and Indian (*Aesculus indica* (Wall. ex Cambess.) Hook.) horse chestnuts. In tissues of red horse chestnut trees, the caterpillars die at the second stage of development [7, 10]. However, we've found unusual conker trees

with markers of resistance to CLM in Kyiv green areas [17]. Over time, plants of this form accumulate small mines on the adaxial surface of leaves. The spatial arrangement and abundance of these mines are typical of CLM's oviposition. Thus, the CLM-resistant form of horse chestnut does not produce specific air-borne repellents, and there are no interruptions of synthesis of attractants, by which the leaf miners find the food resources for caterpillars [9]. Yet, the caterpillars shortly die in leaves of these plants.

The possibility use grafting to study the mechanics of resistance chestnut form founded by the successful experiments of grafting hybrids of the red horse chestnut on two- and three-year old conker trees with varying genotypes [3]. The grafted plants persisted despite certain changes in their metabolism. Thus, the seeds of grafted plants are characterized by stable content of fatty acids (with a high proportion of unsaturated fatty acids, in particular oleic, triterpene saponin escin) and by a highly variable content of flavonols (campferol, quercetin and rutin). Flavonoids of plants of the genus *Aesculus* have antifungal properties [18]. The resistant forms of *Ae. hippocastanum* actively synthesize flavonoid compounds, responding to rupture of leaf tissues because of infection, traumas [15], or phytopathogenic damage [22].

For a long time, the resistance of plants of the genus *Aesculus* was attributed to condensed tannins, the common anti-herbivore defensive mechanism of plants. Condensed tannins (proanthocyanidins) can bind to proteins and inhibit or even stop feeding of pests. Still, the phenol compounds were not effective in plant protection against *Cameraria ohridella* in a number of experimental studies [11, 15, 21]. The opinion that phenol substances, such as proanthocyanidins, are the most important defense mechanism of *Aesculus* plants against herbivores is now under review. Many scientists agree that the total content of phenol compounds and resistance of different *Aesculus* species to *Cameraria ohridella* are not reliably connected. Thus, leaves of the susceptible A*e. turbinata* and of comparatively susceptible *Ae. indica* contain phenols in concentration higher than that in leaves of *Aesculus× neglecta*, which is resistant to *Cameraria ohridella* [4]. Indeed, horse chestnuts are almost unaffected by the majority of pests and remained intact at the beginning of seasonal defoliation of leaves on trees before the spread of CLM in Europe. That is, this provision is well founded, however, the phenolic compounds characteristic of horse chestnuts do not repel CLM. They do not play the role of antifedants and repellents that deter females from laying eggs [5, 11]. It is also possible that plant resistance is related not to the total content of phenols in the leaves, but to the presence of individual compounds [13, 16]. The inefficiency of existing biochemical barriers can be linked to the biological peculiarities of development and nutrition of caterpillars. At the first stage of development, they feed exclusively on cellular juice in epidermal cells containing less tannins than the mesophyll cells [16]. However, this does not explain, how at later stages of development CLM caterpillars consume tannin-rich mesophyll. Is it related to a development of specific enzyme systems later in the pest's life, or do they accumulate bacteria capable of decomposing toxic condensed tannins?

The purpose of this work was to investigate the features of secondary metabolism in leaves of conker trees, resistant to *Cameraria ohridella*, grafted on susceptible two-year-old seedlings, and to find out how changes in metabolism affect the pest impact on the rootstock.

Material and methods. Studies were carried out on grafted and not grafted plants of *Ae. hippocastanum*. The plants selected for the grafting had signs of resistance to horse chestnut miners, according to results of long-term monitoring studies (2010-2018).

As scions we used one-year old shoots of the persistent form of *Ae. hippocastanum*, separated from the mother plants in winter and kept at low temperatures in a humid dark chamber. As rootstock we grew in containers two-year-old seedlings of *Ae. hippocastanum*, susceptible to *Cameraria ohridella*, with a sufficiently developed root system. Shoots of resistant to *Cameraria ohridella* plants of conker trees were grafted on stems of the rootstocks. Grafted plants were kept until beginning of vegetation in the greenhouses of Botanical Garden of the National University of Bioresources and Natural Resorces (NUBiP) of Ukraine. Later, during the leaf formation phase, a number of plants (10 specimens of each kind) were transplanted to the unprotected soil of Botanical Garden of NUBiP.

The physiological state of leaves in the presence or absence of CLM-specific lesions was investigated by the pigment complex, as well as the qualitative and quantitative composition of the phenolic compounds.

Determination of content of carotenoids and chlorophylls a and b in leaves

The pigment content was analyzed in leaves of two-year old seedlings (NRP) before the first signs of CLM damage to the assimilation surface, and in leaves of grafted plants with (GPM) and without (GR) signs of damage to the assimilation surface. Pigments were determined in methanol extracts (ratio of plant material to methanol 1:10). Quantitative analysis of chlorophylls (Chl_a and Chl_b) and carotenoids ($C_{(x+c)}$) was performed at scanning spectrophotometer OptizenPop (South Korea) by the formula:

$$Chl_a\,(mg/ml) = 16.72A_{665.2} - 9.16A_{652.4}$$
$$Chl_b\,(mg/ml) = 34.09A_{652.4} - 15.28A_{665.2}$$
$$Chl_{(x+c)}\,(mg/ml) = (1000A_{700} - 1.63C_a - 104.96C_b) \,/\, 221$$ [7].

Spectrophotometric studies of pigments in leaves of experimental plants were carried out in 4 replicates.

Determination of total phenol content

Phenolic compounds were determined in the medial portions of central leaflets of the palmately compound leaf. The total content of phenolic compounds in leaves was determined spectrophotometrically (OptizenPop, South Korea) with a Folin & Ciocalteu's phenol reagent. The calibration graph was constructed using gallic acid [14].

Determination of flavonoid content

The quantitative content of flavonoids in plant material was estimated spectrophotometrically (OptizenPop, South Korea) at λ = 419 nm. To 300 mkl of extract 200 mkl of 0.1M $AlCl_3$ solution and 300 mkl of 1M CH_3COONa were successively added. The calibration graph was constructed using quercetin (Sigma, Germany).

Determination of catechins content

Catechins content was determined by reaction to vanillin reagent. To 100 mkl of extract 900 mkl of methanol and 2.5 ml of 1 % vanillin solution, and 2.5 ml of $9NH_2SO_4$ methanol solution were successively added. Estimation of the optical density (D) of the reaction mixture was carried out after 30 min at λ = 500 nm [8, 19, 20]. All spectrophotometric studies of the phenolic compounds in leaves of the test plants were performed in 4 replicates.

High-performance thin-layer chromatography (HPTLC)

Qualitative content of plastid pigment was studied using high-performance thin-layer chromatography (HPTLC) on Silicagel G 60 plates (Merck), with the following solvent system: toluene, acetone, formic acid (34:10:2 – v/v/v). The chromatogram was then dried and treated with solution of sulfuric acid in ethyl alcohol and heated for 5 min at 105 °C.

High-performance liquid chromatography (HPLC)

Profiling of secondary metabolites of ordinary chestnut leaves was performed using high-performance diode matrix liq-

uid chromatography (DAD-RP-HPLC) on Agilent 1100 chromatographic system.

We used two eluents (eluent A, 0.05 M aqueous solution of H_3PO_4; eluent B, acetonitrile), column Agilent Poroshell® 120, 2.7 µm, 2.1 × 150 mm at 20 ˚C in thermostat, sample volume 5 µl, eluent flow rate 0.2 ml / min, analysis time up to 80 min, and the following elution profile: broadband linear gradient from 0 % B in A to 100 % B in 30 min, then isocratic B with a flow acceleration to 0.6 ml / min and increasing temperature to 40 ˚C. The detection wavelengths of 206, 254, 300, 350 and 450 nm were chosen to determine most organic compounds (including terpenoids with at least one double bond), most aromatic compounds, phenylpropanoids (mostly cinnamic acid derivatives), flavonoids (mainly flavones and flavonols), carotenoids and chlorophylls, respectively.

The chromatographic data (including absorption spectra) were processed and visualized using Agilent ChemStation and CorelDraw X3 software.

Results and discussion. Grafting of CLM-resistant *Ae. hippocastanum* form (scion) onto stem of susceptible form (rootstock) proved successful. However, only 60–70 % of grafted plants survived. During the first vegetation period, shoot growth was slower compared to shoots of seedlings. Leaflet shape of 25–30 % of surviving grafted plants varied comparatively more (Fig. 1, a–b), bore deformations and mosaic pigmentation, which can be connected to disrupted synthesis and accumulation of plastid and other pigments, such as carotenoids (Fig. 1, c–d).

The quantitative and qualitative composition of plastid pigments, especially chlorophylls, corresponds to the general state of assimilation organs and the ability of plants to maintain a balanced metabolism. The comparative analysis of the pigment complex of grafted plants and CLM-susceptible seedlings of conker trees were compared. The content of chlorophylls was higher in grafted plants compared to seedlings: for basic chlorophyll *a* it was almost twice higher, for chlorophyll *b*, 3.8 times (Table 1).

We think that this fact may be explained by the difference in age of the source material: shoots for the scions were taken from 25–30 year old parent plants. Though, this effect can be related to the specific interaction of the two genotypes.

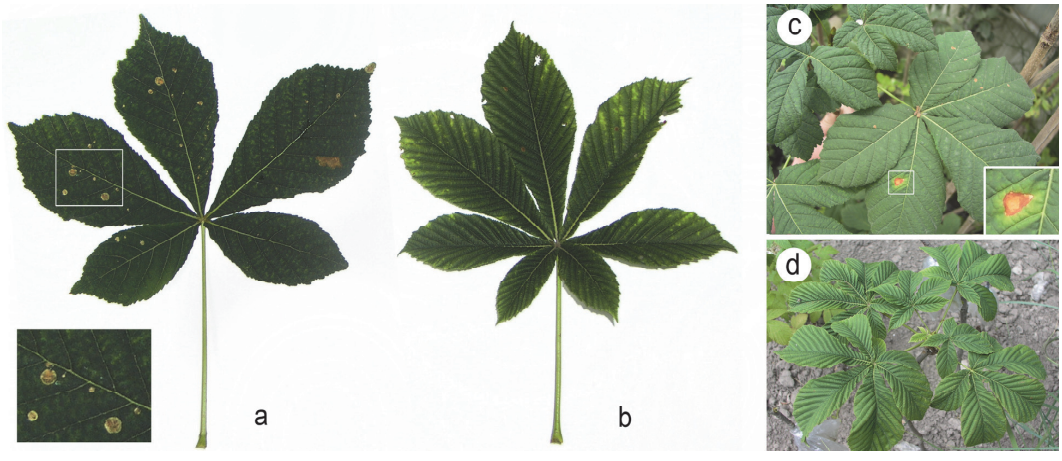


Fig.1. Leaves of CLM–resistant Aesculus hippocastanum form obtained by grafting: a – signs of damage; b – abnormal pigmentation (interstitial chlorosis), without signs of damage; c – abnormal pigmentation around a mine; d – corrugation of leaf.

1. Content of plastid pigments in leaves of experimental conker trees (x ±SE, n = 4)

Plastid pigments	NRP	GP	GPM
Chl a, mg/g	3.50 ± 0.18	6.12 ± 0.31*	7.42 ± 0.37*
Chl b, mg/g	1.15 ± 0.08	4.70 ± 0.32*	3.35 ± 0.20*
Chl a + Chl b (mg/g)	4.64 ± 0.27	10.82 ± 0.76*	10.77 ± 0.75*
Car, mg/g	1.46 ± 0.09	1.43 ± 0.10	2.29 ± 0.21*

Note: NRP – seedling of susceptible form with signs of CLM mines on leaves; GP – grafted seedlings of the 1st type without signs of CLM mines on leaves; GPM – grafted seedlings of the 2nd type with signs of CLM mines on leaves; Chl – chlorophyll; Car – carotenoids; * – values significantly different from NPR ($p < 0.05$).

It is most intriguing that in the middle of vegetation season, signs of CLM damage (mines) began to appear on separate leaflets of grafted plants (Fig. 1, a). CLM caterpillars reached the third stage of development according to the size and shape of mines, at which point the development stopped. Areas with pronounced yellow-brown color appeared around the damaged areas of leaflets, which is characteristic for increasing concentration of flavonoids and carotenoids (Fig. 1, c). This effect correlated with the results of spectrophotometric and chromatographic analyses, which confirmed the significantly increased content of the carotenoid group substances in leaves (Table 1).

The carotenoid content increased by 60 % in leaf tissues damaged by CLM caterpillars. That was accompanied by qualitative changes in the composition of the pigment complex. Two new products with Rf ~ 0.78 and 0.91 with pink fluorescence characteristic of carotenoids were identified on the chromatogram of leaves bearing mines (Fig. 2, c). Since carotenoids have inherent antioxidant properties, it can be assumed that the quantitative increase of pigments of this class is the result of a protective reaction of plants, aimed at reducing the free radical load on cells and organoids. In terms of metabolism, increased carotenoids content in cell is associated with the activation of glycolysis, the end product of which is pyruvic acid. It is converted to acetal-CoA, which is used in the synthesis of isoprenoids, carotenoids and other terpenoids through mevalonic acid and isopentyl pyrophosphate [23].

The densitogram (Fig. 2, c) shows that CLM leaf damage was associated with the significantly increased accumulation of products with Rf ~ 0.68 and 0.73, and the amount of pigment with Rf ~ 0.52 decreased. Hence, one of the reactions of CLM-resistant plants to caterpillar damage is the activation of enzymatic systems involved in the synthesis of terpenoids, and in particular carotenoids.

The formation of mines on the leaves of *Ae. hippocastanum* was accompanied by browning, as well as necrotic damage to the tissues around them. At the same time, the procedure of grafting and the processes of engraftment of tissues with the formation of a common conductive system were themselves linked to significant local and systemic physiological and biochemical rearrangements, which, after adaptation of the plant organism, persisted for two vegetation periods in open soil.

The total pool of phenols in plant leaves after graftage increased slightly and was still maintained at a relatively stable level. Although secondary metabolites play an important role in the formation of plant systemic stability, they did not, however, protect the assimilation organs from CLM damage.

We determined the quantitative content and ratio of different groups of phenolic compounds in leaves of *Ae. hippocastanum*. Reproduction of CLM-resistant forms of *Ae.*

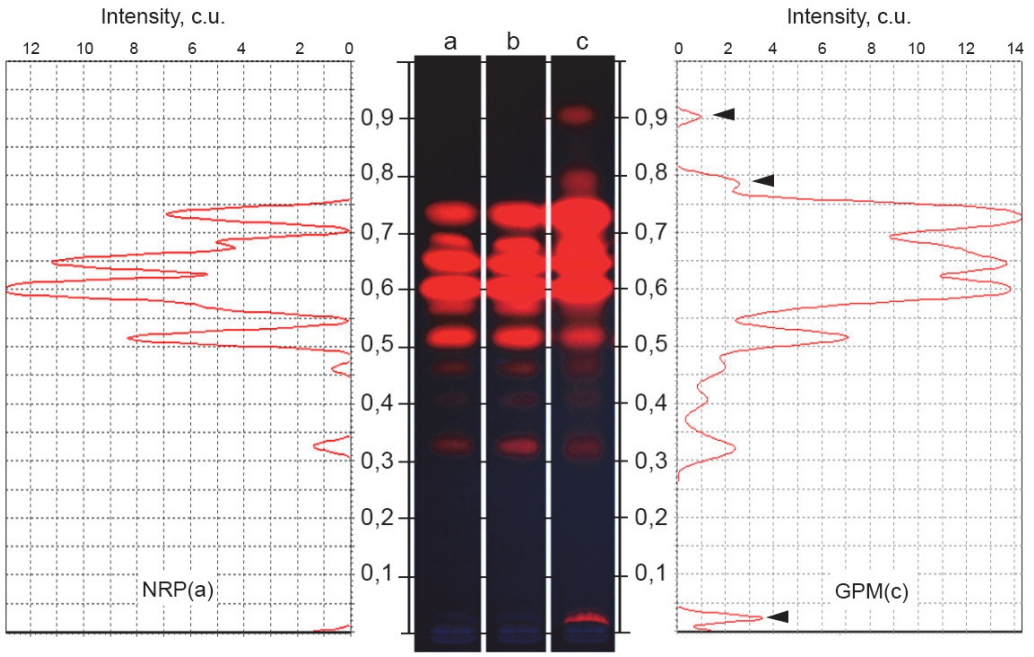


Fig. 2. Chromatography of leaf pigments of conker trees: a – susceptible seedlings with signs of CLM mines on leaves; b – grafted seedlings of type 1 resistant form without signs of CLM mines on the leaves; c – grafted seedlings of type 2 resistant form with signs of CLM mines on leaves.

hippocastanum by grafting to the susceptible rootstock at the beginning of the active growing season resulted in 1.2-1.3 times lower content of flavonoids in the leaves, compared with the original parent plant (Table 2).

Over time, young leaves of grafted plants showed typical signs of CLM damage. The content of proanthocyanidins and catechins increased in leaves (Fig. 3). The divergence in the quantitative indicators of flavonoid content gradually decreased. A definite relationship between the qualitative composition of phenolic compounds and the degree of CLM leaf damage is confirmed [13, 16]. Certain ratio of phenolic compounds is an important factor of plant resistance [12].

The resistant forms of *Ae. hippocastanum* are usually characterized by high content of flavonoids and decrease of total pool of proanthocyanidins.

This confirms the assumed synecological role of the proanthocyanidins of the conker

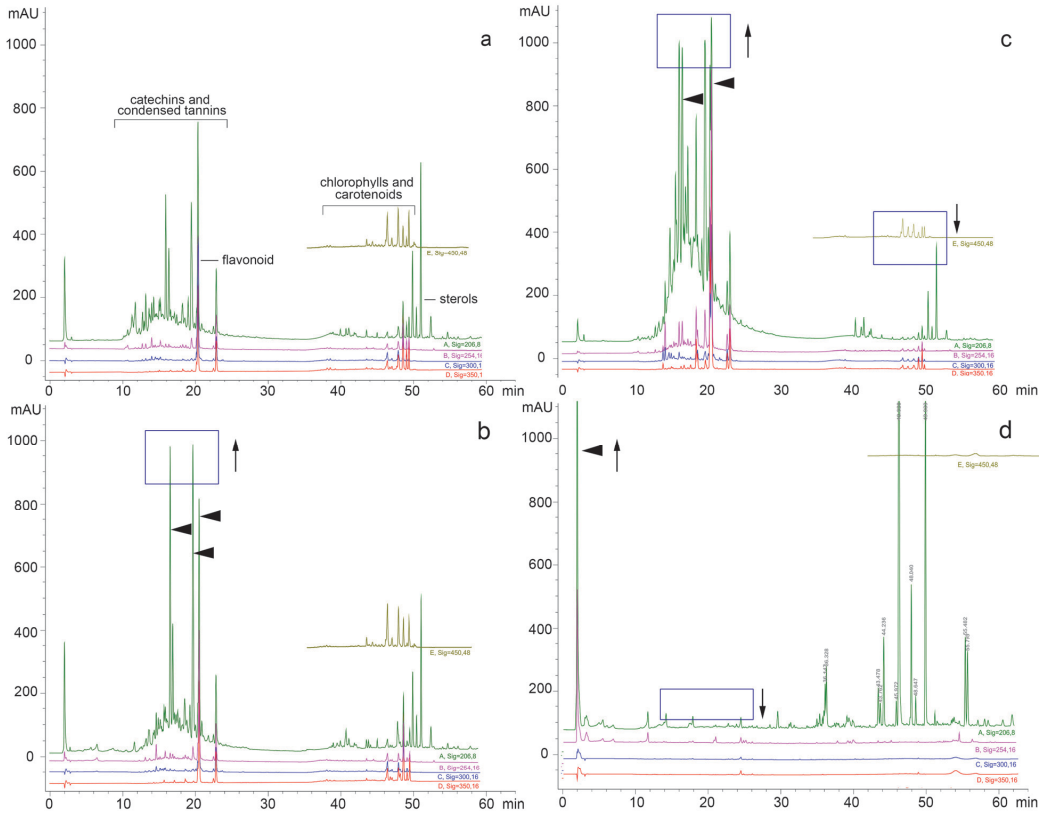
2. Content and ratio of phenolic compounds in leaves of seedlings and grafted plants of conker trees (x ±SE, n = 4)

Form	Phenols, mg/g	Flavonoids, mg/g	Catechins, mg/g	Phenols/Flavonoids
NRP	47.1 ± 2.36	2.7 ± 0.19	31.0 ± 1.55	17.3 ± 0.87
GP	8.3 ± 0.58*	2.4 ± 0.18	14.0 ± 0.98*	3.4 ± 0.17*
GPM	12.1 ± 0.72*	1.8 ± 0.09*	27.6 ± 1.65	6.6 ± 0.33*

Note: legend as given in Table 1; * difference from NRP significant at $p < 0.01$

trees in the formation of interspecies relationships with a rather specialized phytophage. The main source of energy for the caterpillar is hydrocarbons, hydrolyzed in its gut by corresponding enzymes (amylase, maltase, sucrose) [6]. It is shown that in leaves of susceptible chestnut species, the hydrocarbon content is usually higher than in leaves of the resistant ones [5].

HPLC results revealed an increase of two major proanthocyanidins (peaks at 16.2 and 19.5 min) in leaves with mines (Fig. 3, b). It should be noted that the CLM-susceptible form of conker trees is usually (in addition to these two compounds) characterized by a high content of another proanthocyanidin (peak at 20.1 min). This feature of secondary metabolism in the assimilation tissues may be associated with the presence of corresponding enzymes synthesized in rootstock tissues. The enzymes synthesize proanthocyanidins in the presence of a substrate (catechins, leukoanthocyanidins). At the same time, the main phenolic compounds from the *Ae. hippocastanum* leaves are not detected in the intestine and tissues of the caterpillar (Fig. 3, c) indicating active metabolism of phenols. We isolated pure cultures of Gram-negative



3. Chromatogram of methanol extracts of leafs of CLM–resistant *Aesculus hippocastanum* forms, obtained by grafting: not damaged (a) and damaged (b) by caterpillars of *Cameraria ohridella*; leaves with mosaic pigmentation and signs of interstitial chlorosis (c), extract of tissues of *Cameraria ohridella* caterpillar (d); arrows show the tendency to increase (up) or decrease (down) of content of individual compounds in leaves with signs of damage or with pigmentation disorders.

entrobacteria from *Cameraria ohridella* that were able to actively decompose the condensed tannins and phenolecarboxylic acids of *Ae. hippocastanum* leaves, when those were added to nutrient media as aqueous extracts (unpublished data). Therefore, the presence of specific enterobacteria in the gut of CLM caterpillars can contribute to the digestion of condensed tannins, toxic to many phytophages. It is also possible that the isolated enterobacteria are the main producers of enzymes for the fermentation systems necessary for the normal digestion of caterpillars. At the same time, the high concentration of flavonoids (including rutin) with bacteriostatic action is characteristic of resistant species and forms of chestnuts. That can inhibit the bacterial activity in the caterpillar gut, thus increasing the risk of their intoxication.

In addition, the high content of phenolic compounds is undesirable for the plant itself. The leaves of scions with signs of pigmentation disorders also contained a lot of phenolic compounds, in particular condensed tannins. That was accompanied by a significantly deteriorated general condition of the plant. In such leaves the content of chlorophylls and carotenoids decreased (Fig. 3, c).

Therefore, the resistance of conker trees relies a lot on the balance between phenolic and terpenoid synthesis. The group of terpenoids also includes triterpene saponins (in particular escin). The leaves and seeds of *Aesculus* plants are rich in triterpene saponins. D'Costa and Ferracini showed that treating leaves of CLM-susceptible conker trees with triterpene saponins of resistant plants partially reduced the extent of caterpillars damage [2, 12]. That confirms the importance of terpenoid synthesis in the formation of protection mechanisms in plants against CLM.

The synthesis pathways of terpenoids and phenolic compounds are, to some extent, alternative. Most phenolic compounds, in particular oxycoric and oxybenzoic acids are synthrsized via the shikimate pathway [21]. Shikimic acid is formed in the process of condensation of erythrose-4-phosphate and phosphoenolpyruvate molecules and is further transformed into phenylalanine, a key product of plant phenolic synthesis.

Since phosphoenolpyruvate is a penultimate product of glycolysis, the activation of terpenoid synthesis is accompanied by a decrease in its total pool in the metabolic cycle and slows down the synthesis of phenols via the shikimate pathway. There is a group of enzymes involved in the synthesis of flavonoids which is not required for most phenylpropanoids. That enzymatic group can synthesize flavonoids via the acetate-malonate pathway, the starting product of which is also acetyl-CoA. Therefore, the total content flavonoids, carotenoids and other terpenoids is relativelyhigher in CLM-resistant forms of conker trees. That feature of metabolism of CLM-resistant forms of conker trees likely results from the high activity of pyruvate kinase, which provides the transfer of the phosphoryl group from phosphoenolpyruvate to ADP and subsequent synthesis of ATP.

Conclusion. Grafting shoots of the CLM-resistant form of conker trees on two-year-old seedlings of susceptible plants slightly decreases their resistance to the pest. The grafting efficiency is nearly 60-70 %. During the first two years of vegetation, the plants are notably depressed, slow to grow and develop. The leaf shape of the grafted plants is more variable, showing signs of deformation and pigmentation disorders. Partially damaged leaves of the CLM-resistant plants are associated with high concentrations of chlorophylls *a* and *b*. The content and qualitative composition of carotenoids increases and changes in the leaves of these plants, indicating a reorganization of the secondary metabolism associated with the synthesis of phenolic compounds and terpenoids. It is shown that partial damage to the leaves of the CLM-resistant form of conker trees is accompanied by an uncharacteristic increase in the

content of condensed tannins. That confirms the assumption that phenolic compounds of this class do not protect the plant from CLM. It is shown that the caterpillar guts lack phenolic compounds of plant origin, including proanthocyanidins. This is explained by the presence of intestinal enzymes capable of hydrolysis of phenols. The question remains: how does the phenol fermentation occur? Do the caterpillars use their own enzyme systems for this purpose, or does this process involve the enterobacteria from their gut?

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АНОТАЦІЯ

А. Ф. Ліханов, О. В. Колесніченко, І. П. Григорюк, В. П. Грахов, М. Ю. Дубчак, О. Ю. Страшок. *Вплив щеплення на характеристики стійкості гіркокаштана звичайного проти каштанової мінуючої молі (cameraria ohridella deschka & dimić). Біоресурси і природокористування. 2019.* ***11****, №5–6. С.5–14. https://doi.org/ 10.31548/bio2019.04.001*

Анотація. *Досліджено взаємовплив підщепи та прищепи на збереження ознак стійкості рослин гіркокаштана звичайного (Aesculus hippocastanum L.) проти молі каштанової мінуючої (КММ) (Cameraria oridella Deschka and Dimić). Встановлено, що ефективність отримання посадкового матеріалу щепленням складає 60-70 %. З'ясовано, що після щеплення пагонів стійкої проти КММ форми гіркокаштана звичайного на дворічні, які не стійкі проти КММ рослини гіркокаштана звичайного, резистентність прищепи проти фітофага знижується. У перші два роки вегетації за підвищеною концентрацією хлорофілів a й b та каротиноїдів листки щеплених рослин набувають ознак деформації і часткового пошкодження КММ. У листках рослин з мінами КММ підвищується кількість фенолів і катехінів, що свідчить щодо перебудови вторинного метаболізму, який пов'язаний з синтезом фенольних сполук та терпеноїдів. Часткове пошкодження листків стійкої проти КММ форми гіркокаштана звичайного на фоні підвищеного вмісту конденсованих танінів підтверджує їхню функціональну неспроможність захищати рослини від КММ. Показано, що гусениці КММ здатні до ферментного гідролізу фенолів, зокрема проантоціанідинів. На підставі наявних експериментальних даних висловлено припущення щодо можливості ферментації фенолів у кишковику гусениць КММ за участю ентеробактерій.*

Ключові слова: *Гіркокаштан звичайний, прищепа, підщепа, стійкість, міль каштанова мінуюча, феноли, таніни, каротиноїди*