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Modification of the epicuticular waxes of plant leaves due to increased sunlight intensity

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Climatic changes observed around the world in recent years are associated with an increase in the solar radiation intensity and temperature and reduction in the humidity. Fluctuations of environmental factors significantly change the conditions for the existence of plants, which dictates the need for adaptive reactions of plant organisms at the different levels of their organization. Such dangerous processes as excessive heating of the surface of plant leaves and water loss can be prevented by the formation of a cuticle, which is a complex composition consisting of cutin and the soluble intracuticular and epicuticular waxes. We suggested that the structure, component composition and properties of the cuticle of trees undergo adaptive changes due to microclimatic conditions in different parts of the tree crown. The study was aimed at the identification and evaluation of light-induced differences in the accumulation and composition of leaf epicuticular waxes of *Ulmus* trees (native *U. minor* Mill. and alien *U. pumila* L.), and was conducted in 2018–2019 in Dnipro city located in the steppe zone of Ukraine. Analysis of the waxes' chloroform extracts was carried out using GC Shimadzu 2010 PLUS equipped with a flame ionization detector and capillary column SP-2560. The highest amount of epicuticular waxes ($12.23 \pm 0.39 \mu\text{g}/\text{cm}^2$) was on the sunlit leaves of *U. pumila*, and wax deposits on the sunned leaves exceeded twice those on the shaded leaves in both *U. minor* and *U. pumila*. Long-chain hydrocarbons detected in the epicuticular waxes of both elm species were represented by free fatty acids, aldehydes, alcohols, and n-alkanes in various ratios. In the epicuticular waxes of *U. minor*, fatty acids dominated both on shaded and sunned leaves, while alkanes together with alcohols were the main components in *U. pumila* waxes, especially on the sunlit leaves. According to our results, local high illumination of leaves in the crown of both elm species caused increase in share of long-chain alkanes (1.2–1.9 times), but simultaneous reduction of the content of free fatty acids (1.5–16.8 times) in the epicuticular waxes' composition. General patterns of the leaf epicuticular waxes' modification due to increased solar radiation and air temperature can indicate the adaptive metabolic responses of woody plants to changing climatic conditions.

Keywords: elm; cuticular wax; hydrocarbon composition; steppe climate; metabolic adaptation.

Introduction

Climate is the key environmental factor which determines the ontogeny of plants and their geographical distribution (Ramirez-Valiente et al., 2015), so any variations in climate entail changes in plant growth and productivity. Today, one of the urgent tasks of ecology is to find the ways that ensure the maintenance and rational use of biological diversity and productivity of ecosystems (Didur et al., 2018) including phytocenoses (Klymenko et al., 2017; Shcherbyna et al., 2017; Khromykh et al., 2018a, 2018b) and zoocenoses (Didur et al., 2019; Lykholat et al., 2019; Pokhlyenko et al., 2019), as well as improving human health (Pertseva et al., 2012; Lykholat et al., 2016).

Plant organisms, due to their attached lifestyle, are permanently exposed to abiotic and biotic environmental factors that reflect on their state (Nazarenko & Lykholat, 2018; Nazarenko et al., 2018). During the last decades, climatic changes observed in all the regions of the world are associated with an increase in the solar radiation intensity and temperature and simultaneous reduction in humidity (Bussotti et al., 2015; Lykholat et al., 2018b). Due to such circumstances, heating of the leaf surface and increasing water loss become inevitable dangerous effects, which are countered by the cuticle. Studies conducted in recent years confirm the role of the cuticle as one of the most significant adaptations of plants to life in the atmospheric environment (Jetter & Reiderer, 2016). The restriction of the non-stomatal transpiration is the most important physiological function of the cuticle (Buschhaus et al., 2007). In addition, the cuticle plays a role in minimizing the adhesion of dust, protecting tissues from UV radiation, microbes and insects (Müller & Riererer, 2005), powdery mildew fungus (Hansjakob et al., 2010), osmotic stress

and pollution (Shepherd & Griffiths, 2006), and preventing deleterious fusions between different plant organs (Tanaka & Machida, 2013).

Cuticle is formed by plant epidermis and considered not as a physical barrier only, but as a type of cell wall modification mainly composed of a three-dimensional network of the polyester matrix (cutin) and the soluble cuticular waxes (Reina-Pintoa & Yephremov, 2009). The cuticular waxes of different plant species contain free fatty acids, primary and secondary alcohols, aldehydes, mono-acid esters of high-molecular alcohols, and also homologous series of long-chain aliphatic compounds represented by n-alkanes of chain length from C_{20} to C_{36} , and alkyl esters from C_{38} to C_{70} (Jetter & Riederer, 2016). The cuticular wax can be embedded in the cutin polymer (called intracuticular wax), or be on the outer surface of the cuticle (epicuticular wax). In wild-type plants, triterpenoids and other cyclic compounds, as well as the primary alcohols, preferentially accumulate within the intracuticular wax (Buschhaus et al., 2007), while a mixture of long-chain aliphatic molecules including free fatty acids and alkanes in many cases accumulate in the epicuticular layer (Buschhaus & Jetter, 2011).

Formation of the transpiration barrier is provided mainly by cutin and intracuticular wax, while contribution of the epicuticular waxes in this case is assessed as not very significant (Zeisler & Schreiber, 2016). However, an important role of the epicuticular waxes in the life of plant organisms was proved experimentally in 24 plant species, which in a short time restored the outer layer of waxes, previously removed from the surface of plant leaves (Neinhuis et al., 2001). The natural plant resistance to the damaging effect of insects can be provided by epicuticular waxes, indicating their participation in the defense against biotic stresses (Bohinc et al., 2014; Brygadyrenko & Nazimov, 2014; Brygadyrenko, 2016).

Epicuticular waxes often form two- and three-dimensional structures, which influence the wettability, self-cleaning behaviour and the light reflection at the cuticle interface (Grant et al., 2003; Wen et al., 2006; Koch & Ensikat, 2008). As a result, plant response to the impact of drought, light, high temperature and other abiotic environmental factors can be accompanied by an increase in the amount of epicuticular waxes' deposits and their appearance (Kim et al., 2007). However, the conditionality of wax composition due to climatic factors has been little studied. In previous works, we established the interconnection of mass and component composition of the epicuticular waxes with local illumination level of leaves in the crown of various linden species (Lykholat et al., 2017, 2018a). Now we proceeded from the position that response of any plant organism to changing environmental conditions is based on the evolutionarily formed adaptive mechanisms. It was hypothesized that the mass and composition of epicuticular waxes synthesized by any plant species at various local levels of illumination and temperature may indicate the plant's adaptation to actual microclimatic conditions. The objective of the present study was to reveal the patterns of change in the epicuticular wax accumulation as well as in composition of wax hydrocarbons depending on leaves' illumination level in the crown of elm plants.

Materials and methods

The study was conducted in 2018–2019 in Dnipro city, within the steppe zone of Ukraine (Fig. 1). The climate of the region has distinct continental features, including seasonal droughts with high temperatures and dry hot winds. A small average amount of precipitation (472 mm) decreases in arid years to 250 mm, and the total evaporation for a year exceeds the amount of precipitation by 2–3 times. Here, the woody plants grow in the conditions of ecological mismatch, and show high sensitivity even to small changes in the climatic factors (Khromykh et al., 2018a), as well as to anthropogenic pollution (Alexeyeva et al., 2016).

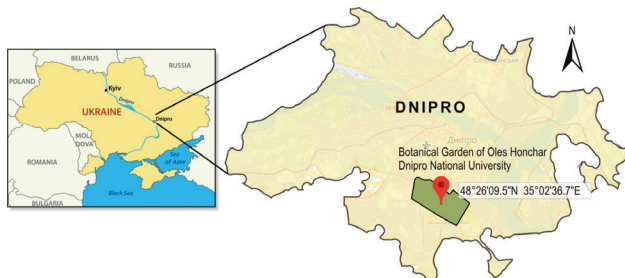


Fig. 1. Location of the Botanical Garden of Oles Honchar Dnipro National University on the territory of Dnipro city

The test objects were the sun-adapted and shade-adapted leaves of two species of the genus *Ulmus* L. Samples were selected in the Botanical Garden of Oles Honchar Dnipro National University, taking into account the relatively low pollution of this urban area. The autochthonous elm species (*U. minor* Mill.) is spread over almost the entire territory of Ukraine as a park and anti-erosion tree culture. The alien species (*U. pumila* L.) originated from the South Asian region and was introduced in the steppe zone in the middle of last century. Plant leaves were selected from 5–7 trees of each species during the full development of leaf surface (in July) in sunny weather in the middle of the day. The leaves adapted to sunlight were taken along the perimeter of the crown, and the leaves adapted to the shadow – inside the crown of trees, both at 2.0–2.5 m.

The epicuticular waxes from leaf surface were extracted with chloroform in accordance with the method of Buchhaus et al. (2007). Briefly, each cut leaf fragment having an area of 1 cm² was immersed in the solvent for 30 s, after which the total extracts were dried in a stream of nitrogen. The wax amount was calculated by the weight method and expressed in µg per unit leaf area.

The method of capillary gas chromatography was applied to study the composition of hydrocarbons in the elm epicuticular waxes. Analysis of the chloroform extracts of waxes was carried out using GC Shimadzu 2010 Plus equipped with a flame ionization detector (FID) and capillary column SP-2560. Sample in a volume of 1.0 µL was applied

to a column containing bis(cyanopropyl)polysiloxane as a fixed liquid phase and having a length of 100 m. The programmed temperature gradient from 100 to 230 °C was increased at a rate of 10 °C per minute. The quantitative content of individual hydrocarbons was determined from the peak area and retention times of the components on chromatograms processed by the internal normalization method. Content of the individual wax components was expressed as a percentage of total amounts.

Average samples of plant leaves were prepared in triplicate. The wax extraction with chloroform and gas chromatography analysis were performed for each sample. The average amount of wax deposits as well as the hydrocarbon's content are expressed as the mean ± standard deviation ($\bar{x} \pm SD$). The analyzed parameters were processed using variance method (ANOVA) factorial experiment, and differences were considered to be statistically significant at $P < 0.05$.

Results

The total amount of waxes determined in the chloroform extracts from the surface of illuminated and shaded leaves was different in both elm species (Table 1).

Table 1
Epicuticular wax accumulation on the elm leaves depending the level of illumination in the crown of trees ($\bar{x} \pm SD$, n = 40)

Plant species	Mass of the epicuticular waxes, µg/cm ²	
	shaded leaves	sunlit leaves
<i>U. minor</i>	2.4 ± 0.1 ^{***}	6.3 ± 0.2
<i>U. pumila</i>	7.8 ± 0.3 ^{***}	12.2 ± 0.4

Note: *** – significance $P < 0.001$.

Accumulation of the epicuticular wax deposits on the surface of sun-adapted leaves significantly exceeded the indices for shaded leaves in both *Ulmus* species (1.6 and 2.6 times, respectively for *U. pumila* and *U. minor*, $P < 0.05$).

In this paper, the typical chromatograms (one of at least three similar) of the long-chain hydrocarbons detected by GC method in the chloroform extracts of the epicuticular wax are presented. Epicuticular waxes from the surface of shaded (Fig. 2a) and sunlit (Fig. 2b) leaves of *U. minor* had qualitative and quantitative differences in the composition of hydrocarbons.

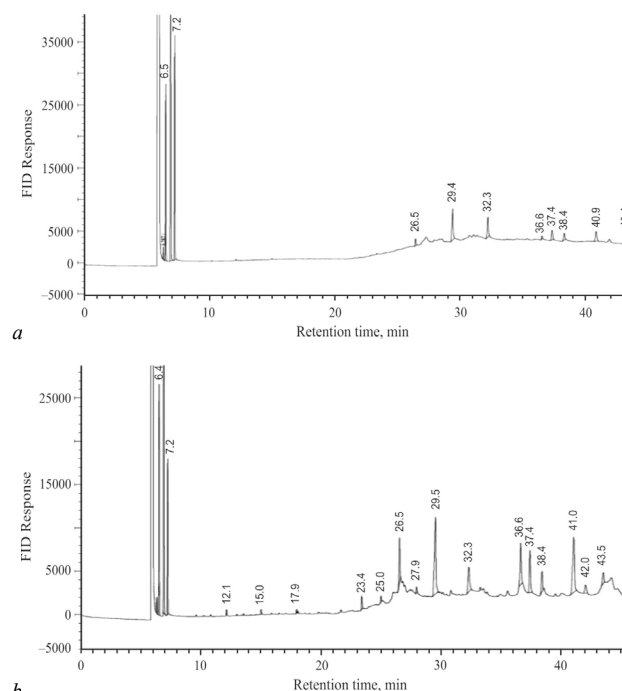


Fig. 2. GC analysis of the epicuticular wax hydrocarbons of *U. minor* shade-adapted (a) and sun-adapted (b) leaves: the peak area is indicated along the ordinate axis; above the peaks, the retention time of the individual components is indicated

GC analysis of the component composition of *U. pumila* epicuticular waxes revealed a similar light-dependent redistribution of hydrocarbons in the shaded (Fig. 3a) in comparison with sunlit (Fig. 3b) plant leaves.

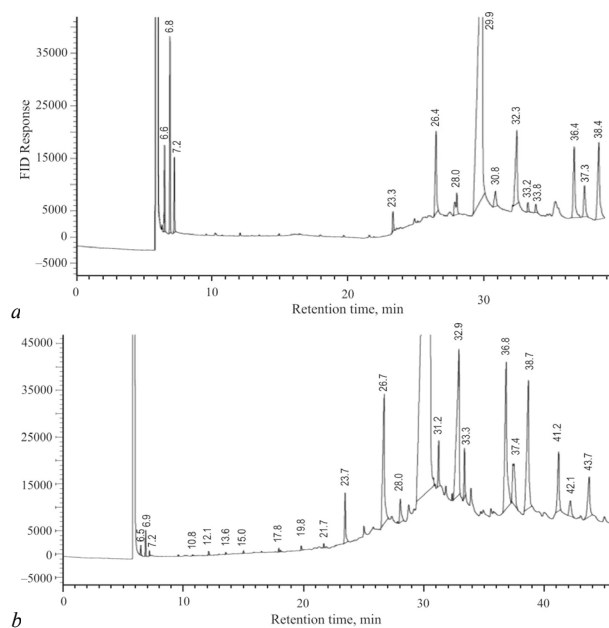


Fig. 3. GC analysis of the epicuticular wax hydrocarbons of *U. pumila* shade-adapted (a) and sun-adapted (b) leaves: the peak area is indicated along the ordinate axis; above the peaks, the retention time of the individual components is indicated

The arrangement of the different classes of hydrocarbons in the chromatograms was established, referring to numerous published data on the component composition of the epicuticular waxes from the leaf surface of different plant species. As a result, the hydrocarbon components of epicuticular waxes of the elm leaves were represented by a large share of the very long-chain fatty acid derivatives (VLCHA) with the predominance of alkanes (C₂₉–C₃₄), and the smaller proportions of primary alcohols (C₂₇–C₂₈), aldehydes (C₂₅–C₂₆), and free fatty acids (C₁₆–C₂₄).

Analysis of chromatograms obtained showed that the long-chain hydrocarbons in the epicuticular waxes of both elm species were represented by the same classes of aliphatic compounds that were combined into three groups. The ratio of the components in the epicuticular wax of sunlit and shaded *U. minor* leaves varied markedly, especially within classes of aldehydes, alkanes and alcohols (Table 2).

Table 2
Hydrocarbons classes' content (% of the total) in the epicuticular waxes of *U. minor* leaves (x ± SD, n = 5)

Type of plant leaf	Free fatty acids	Aldehydes	Alkanes and alcohols
Shaded leaves	77.1 ± 1.8***	1.2 ± 0.2***	21.7 ± 0.8***
Sunlit leaves	52.4 ± 1.5	6.2 ± 0.3	41.4 ± 1.4

Note: *** – significance P < 0.001.

In the epicuticular waxes of *U. minor* shaded leaves, free fatty acids dominated indisputably, while in sunlit leaves the share of fatty acids decreased 1.5 times, when the contribution of aldehydes and the sum of alkanes and alcohols increased (2.9 and 1.6 times, respectively). As for the hydrocarbons composition of the epicuticular waxes of *U. pumila* plants, most significant differences between the sunlit and shaded leaves were in the content of free fatty acids (Table 3). In waxes from both the shaded and sunlit leaves of *U. pumila*, alkanes together with alcohols were the main classes of long-chain hydrocarbons, and the share of these components in the waxes of sun-adapted leaves was 1.2 times higher.

Discussion

The total mass of epicuticular waxes accumulated on the plants leaves is a dynamic indicator, since it changes during ontogenesis (Kim et al.,

2009). Epicuticular wax deposits measured on leaves of both *Ulmus* species (Table 1) coincide with the level of epicuticular waxes' accumulation on leaf surface of different wild-type plants in the range of 5–30 µg/cm² (Buschhaus & Jetter, 2011). The study results are also close to the level of waxes on the leaf surface of eight plant species from different regions of origin, which ranged 3.0–16.0 µg/cm² (Jetter & Riederer, 2016). At the same time, the epicuticular wax amount of both elm species exceeded the wax deposition 1.7 µg/cm² on *Phyllostachys aurea* leaves (Racovita & Jetter, 2016) and the wax deposits in a range of 0.9–1.5 µg/cm² on adaxial surface of apple tree leaves (Bringe et al., 2006). So, rather large total mass of the epicuticular wax deposits and their predominant accumulation on the sunlit leaves were the common patterns to both elm species, which is consistent with previously studied epicuticular waxes of the genus *Tilia* plants, including *T. tomentosa* (Lykholat et al., 2017) as well as *T. cordata*, *T. platyphyllos* and *T. begonifolia* (Lykholat et al., 2018a).

Table 3
Hydrocarbons classes' content (% of the total) in the epicuticular waxes of *U. pumila* leaves (x ± SD, n = 5)

Type of plant leaf	Free fatty acids	Aldehydes	Alkanes and alcohols
Shaded leaves	10.1 ± 0.3***	9.8 ± 0.3*	80.1 ± 1.8***
Sunlit leaves	0.6 ± 0.1	7.7 ± 0.9	91.7 ± 2.1

Note: * – significance P < 0.05, *** – P < 0.001.

According to the data of Grant et al. (2003), high reflectance of tree leaves was likely due to the presence of various epicuticular wax structures on the leaf surface. We hypothesized that increased epicuticular wax layer of both *Ulmus* species can enhance the reflectivity of the leaf surface and facilitate the plants' adaptation to the local intensive solar radiation. In favour of this assumption is data that the barley genotypes having a higher tolerance to drought and yield as well, were characterized by a higher level of wax deposits on the leaf surface (González & Ayerbe, 2010).

The hydrocarbon components of epicuticular waxes extracted from the surface of leaves of both elms belonged to several different classes, which is consistent with the literature (Jetter & Reiderer, 2016; Zeisler & Schreiber, 2016; Engelsdorf et al., 2017). Analysis of the study results suggests that an increase in the proportion of aliphatic components with a longer chain in the epicuticular waxes of sun-adapted leaves can be considered as a general pattern for both *U. minor* (Fig. 2) and *U. pumila* (Fig. 3). The results obtained coincide with the data on dominance of long-chain alkanes in the epicuticular waxes of *Sesamum indicum* (Kim et al., 2007) and *Kalanchoe daigremontiana* (Van Maar-seveen & Jetter, 2009). A similar increase in the share of hydrocarbons with a longer chain length was found earlier in the waxes of sunlit leaves of *T. tomentosa* (Lykholat et al., 2017) and *T. cordata*, *T. platyphyllos*, and *T. begonifolia* (Lykholat et al., 2018a) as well. The decrease in proportion of free fatty acids in the epicuticular waxes of sunlit leaves of both elm species (Table 2, 3) is another general trend, which was most sharply manifested in the case of *U. pumila* waxes (a drop of 16.8 times).

The light-induced changes in elm leaf epicuticular waxes, as well as a previously identified similar shift in the linden leaf waxes (Lykholat et al., 2017, 2018a), indicate the ability of plants to regulate effectively the composition of the cuticle outer layer depending on environmental conditions. Such modifications of waxes, including changes in the biosynthesis of hydrocarbons with longer chain, undoubtedly have an adaptive value for plants. This assumption is supported by the published data about positive correlation between the amount of long-chain alkanes in the epicuticular waxes and plant resistance to adverse effects. For instance, predominance of n-alkanes and aldehydes in the epicuticular wax composition contributes to a greater resistance of the abaxial surface of *Lolium perenne* leaves to powdery mildew in comparison with adaxial surface, where primary alcohols and esters prevailed (Ringelmann et al., 2009). Studies of experimental water deficiency attribute the increase in resistance of *Arabidopsis thaliana* plants to the increase in wax alkanes number (Kosma et al., 2009). The age-induced changes in the epicuticular waxes' composition of *Malus domestica* leaves consisted of an increase in proportion of alcohols, esters and alka-

nes, while the share of fatty acids decreased (Bringe et al., 2006). The patterns of light-induced shift in leaf epicuticular waxes of *U. minor* and *U. pumila* are consistent with the notion that biosynthesis of long-chain alkanes of the surface waxes depends on the influence of environmental factors (Kunst & Samuels, 2009; Domínguez et al., 2011). Trends of the *Ulmus* species epicuticular wax changes confirm the concept of a complex regulatory network for control of cuticle synthesis (Yeats & Rose, 2013) and also the interpretation of cuticle as the outer zone of epidermal cell walls of plants (Nobusawa et al., 2013; Guzmán-Delgado et al., 2016). The results of our study support the view of Guo et al. (2015) that accumulation of surface waxes can be considered a universal plant response to environmental changes.

It must be noted that the epicuticular waxes both of shaded and sunlit leaves of the Asian species *U. pumila* had the much higher percentage of long-chain alkanes than the respective waxes of the autochthonous species *U. minor*. This difference may be one of many possible reasons for the greater adaptability of alien plants from southern areas to intensification of solar radiation. The present results are in the same line with previous findings (Khromykh et al., 2018a, 2018b; Lykholat et al., 2018b) that some introduced southern plant species have received advantages for vegetation and distribution in the steppe zone under the influence of climate changes during recent years.

Conclusions

Significant differences in the total amount and hydrocarbons composition of the epicuticular waxes of *Ulmus* species arose due to the arrangement of leaves in the tree crown. Epicuticular wax accumulation was much greater in sunned leaves; especially in case of *U. pumila*. The smallest epicuticular wax deposition was found on the shaded leaves of the native species *U. minor*, while the highest was on the sunlit leaves of the alien species *U. pumila*. The share of long-chain n-alkanes together with alcohols increased in the waxes of sun-adapted leaves of both elm species, though it remained noticeably larger in waxes of *U. pumila*. The general pattern of the epicuticular waxes change established might be associated with the plants' adaptation to the solar radiation and temperature enhancement, and climate change on the whole. It seems likely that more significant increase in total wax accumulation, shift in hydrocarbon classes and chain length in *U. pumila* epicuticular waxes could lead to the greater adaptive ability of the alien species.

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