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Changes in leaf epicuticular wax with age of *Chenopodium album* L. and *Abutilon theophrasti* Medik

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Abstract: Epicuticular wax comprises a complex mixture of diverse organic compounds. The predominant class of compounds consists of long-chain *n*-alkanes. *Chenopodium album* and *Abutilon theophrasti* are cosmopolitan weed species, both of economic importance due to difficulties in control, and both species produce wax on the leaf surface. This study shows that in *C. album*, the proportion of epicuticular waxes has higher values in the oldest leaves and lower in the youngest leaves. Conversely, in *A. theophrasti*, the mean wax content tended to be slightly higher in the younger upper leaves compared to the lower leaves. The proportion of waxes in leaves does not reflect the stage of development in either species. In the epicuticular wax composition of *C. album* leaves, alkanes and alcohols are the most abundant compounds. Conversely, in *A. theophrasti* leaves, alkanes, alcohols, and triterpenes dominate. Quantitative variations in leaf epicuticular waxes are influenced by leaf age.

Keywords: weed; leaf characteristics; stage of development.

INTRODUCTION

Chenopodium album L. (lambsquarters) and *Abutilon theophrasti* Medik. could be considered as part of a group of the most common weed species that can be found in arable crops across the globe.[1,2,3] These two species have different leaf surface morphology which is significant not only for taxonomy but also to the herbicide efficacy due to the uptake and effectiveness of herbicides. *C. album* produces wax on the leaf surface, which is a barrier to the absorption of herbicides,[4] while *A. theophrasti* has a high number of trichomes on both sides of leaves,[5] and also certain amount of wax is present on its leaves.[6]

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The epicuticular together with the cuticle, protect the plant's integrity and act as a barrier against biotic and abiotic stresses.[7] Beyond the epicuticular wax that resides on the leaf surface, the cuticle typically harbors an inner layer of wax. This intracuticular wax, oriented perpendicular to the cuticle surface, is embedded within the cutin matrix. Within the very-long-chain aliphatic wax components, primary alcohols tend to accumulate to higher percentages in the intracuticular wax layer, while free fatty acids and alkanes in many cases accumulate in the epicuticular layer.[8]

Epicuticular wax comprises a complex mixture of diverse organic compounds. The predominant class of compounds consists of long-chain *n*-alkanes, characterized by a carbon chain length ranging from C_{27} to C_{31}. In addition to alkanes, other chemical constituents are present in wax, including unsaturated hydrocarbons, alcohols, aldehydes, ketones, acids, esters, and other related compounds. Furthermore, cyclic compounds such as triterpenes, hydroxycinnamic acid derivatives, sterols, and flavonoids can also be found in epicuticular wax.[9] A thin-layer chromatography (TLC) was used to analyze the epicuticular wax composition of *A. theophrasti*.[6] In this findings it was revealed that this wax consists of fatty acids, primary alcohols, secondary alcohols, esters, and hydrocarbons. Notably, the chemical composition of cuticular wax undergoes changes during the ontogenetic development of leaves.

Surface waxes can be effectively analyzed using a combined approach involving gas chromatography (GC) and mass spectrometry (MS).[10] On the other hand, due to the volatile nature of wax compounds, GC/MS are well-suited for identifying and characterizing constituents within wax mixtures.[9,11] Additionally, gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) revealed that *n*-alkanes constitute an average of approximately 10-12 % in *Pinus heldreichii* var. *Pančići Fukarek*.[12]

Epicuticular waxes form a critical barrier on the surface of leaves, with roles in limiting non-stomatal water loss, modulating interactions with pathogens, and influencing the retention and penetration of foliar-applied agrochemicals. Numerous studies on crop plants and woody perennials have shown that both the quantity and composition of surface waxes change substantially during leaf development. In *Sorghum halepense* L., for example, young expanding leaves typically exhibit lower wax loads enriched in primary alcohols and aldehydes, whereas mature leaves accumulate greater amounts of long-chain alkanes and cyclic components, resulting in more crystalline and hydrophobic surfaces.[13] In maize, alkanes and alkyl esters are dominant components of cuticular waxes of adult plant leaves. During plant development a switch from alkanes to esters as the major wax type, which is parallel with emergence of an osmiophilic layer of the cuticle, resulting in establishment of the water barrier.[14] Similar ontogenetic shifts have been reported in *Arabidopsis* leaves, where the amount and composition of

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the cuticular wax mixture change as organs.[15] These developmental trajectories highlight a strong age dependence of wax deposition and structure, with potential consequences for leaf surface physiology.

In the context of weed science, investigations into epicuticular waxes have primarily addressed morphological traits, environmental stress responses, or herbicide droplet behavior, but age-related analyses are limited. For weed species, including *C. album* and *A. theophrasti* as two widespread weeds in temperate agroecosystems, information about wax changes with leaf age are missing. This represents a significant gap, given that surface waxes are known to be a major determinant of foliar herbicide uptake and biological activity.[6] By systematically characterizing wax traits across a multi-age ontogenetic series under controlled growth conditions, the present study addresses this knowledge gap and provides an improved understanding of how developmental dynamics in surface waxes may influence herbicide performance in these two problematic species.

The study aims to assess the qualitative and quantitative wax content in the leaves of *C. album* and *A. theophrasti*. In particular to determine whether there are differences in the content of wax in different maturity phases of leaves.

EXPERIMENTAL

Plant material

The seeds of *C. album* and *A. theophrasti* were collected at the end of September from the agricultural field (44°47′0.8″ N, 20°17′44.9″ E), near Belgrade, Serbia, and stored at room temperature in the dark until planting. Plants were grown in growth chambers. Temperature was set to 27 °C during daylight hours (14 h), and 22 °C during darkness (10 h), with supplemental light intensity of photosynthetic photon flux density (PPFD) ≈ 1300 µmol m^{-2} s^{-1} (MH Philips 600 W) at 1 m height. The plants were grown in plastic pots (diameter 10 cm) with 300 ml volume, containing a commercial potting mix (Floragard TKS1®, pH value of 5.6 with content N 140 mg L^{-1}, P 80 mg L^{-1}, K 190 mg lL^{1}, Germany). Weed seeds were scattered on the surface of soil, and then covered with 1 cm of potting mix. Pots were afterwards watered to near full water capacity and subsequently watered daily to maintain adequate soil moisture for plant growth. Emerged plants were thinned to a density of 2 uniformly sized plants per pot. When *C. album* reached 5 pairs of leaves and *A. theophrasti* reached 5 leaves, leaves samples for wax contend analyses were harvested and storage at -20 °C.

The content of wax in the leaves of C. album and A. theophrasti

The research focused on changes in the chemical composition of epicuticular waxes in different growth stages of *C. album* and *A. theophrasti leaves*: *C. album* at stage 10 (5 pairs) and *A. theophrasti* at stage 5 of leaf development. To ensure consistency, leaves from both species were divided into five subsamples (related to five position), with three replication (weighing approximately 1 g are taken from each phase). Each replicate consisted of pooled leaves from four plants per pot (one pot = one replicate). For each species and each of the five leaf positions, three biological replicates (n=3) were analyzed. These samples of leaves were to be of comparable size between stages. Leaf/leaf pairs (1–5) were defined as consecutive opposite leaves counted from the base of the stem (1 = oldest fully expanded leaves at the lowest node; 5 = youngest fully expanded leaves near the apex). This morphological criterion was

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applied equally to both species to ensure comparability. Prior to analysis, the samples had been carefully packed in aluminum foil and stored at -20 °C. Epicuticular waxes were extracted from the leaf surfaces using 20 ml of hexane. The extraction process involved gentle shaking for 1 minute, followed by filtration through filter paper. Since the goal of was to isolate epicuticular waxes, non-polar hexane as the solvent and a short extraction time of 1 min were chosen. The resulting extract was then dissolved in CH_2Cl_2 and subjected to analysis using gas chromatography-mass spectrometry (GC/MS).

The GC/FID (gas chromatography with flame ionization detection) and GC/MS analyses were conducted using an Agilent 7890 A instrument equipped with an automatic injection system (Agilent GC Sampler 80), an Agilent 5975C XL EI/CI quadrupole mass detector (MSD), and an HP-5 MS 5% Phenyl Methyl Silox capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness). The temperature program involved a linear increase from 60 to 300 °C at a rate of 3 °C min^{-1}. The injector temperature was set at 250 °C, the detector temperature at 300 °C, the source temperature at 230 °C, and the quadrupole temperature at 150 °C. Helium served as the carrier gas (16.255 psi, in constant pressure mode). Samples (1 µl) were injected in splitless mode (using Agilent splitless liner PN 5190-2292) with 1.5 min splitless purge time. Splitless purge time: 1.5 min with 150 mL/min, then gas saver mode (20 mL/min) activate after 2.0 min. Liner type: splitless liner ID 4 mm, Agilent Technologies (Agilent inlet liner, Ultra Inert, splitless, single taper product number 5190-2292). Inlet pressure 16.255 psi, in constant pressure mode, He currier gas. Initially runs split 10:1 was used, and based on quality control check, it was necessary to use splitless mode to increase sensitivity and reproducibility. Moreover, n-alkanes C8-C40 (even series) were used to check sensitivity and to ensure that high-boiling compounds were able to reach detector during GC-MS run.

Electron ionization (EI-MS; 70 eV) was used to obtain mass spectra, and ion detection occurred within the range of 30-550 mz^{-1}. Compound identification relied on comparing EI mass spectra with those from the Wiley and NIST libraries using NIST MS Search 2.0 and AMDIS software. Additionally, calculated retention indices (RI) were compared with library retention indices. Based on the obtained results, the relative proportions of each component in individual samples were determined. The relative percentage of the epicuticular wax constituents was calculated as percentage of normalization of FID peak area. Wax quantities were expressed relative to fresh leaf mass.

Statistical data analysis

The statistical analysis of the collected data was performed using the software STATISTICA® 7.0 (StatSoft, Inc., 2007), a comprehensive data analysis system. For each species and each leaf position, three biological replicates (n=3) were analyzed. One-way ANOVA was applied to test differences in leaf epicuticular waxes content between positions. Prior to ANOVA, all data were tested for normality (Shapiro-Wilk test, $P > 0.05$) and homogeneity of variances (Levene's test, $P > 0.05$). Data with unequal variances were log (x+1) transformed to meet the assumption of homogeneity of variances. Specifically, we used either the LSD test or the t-test for our statistical comparisons. In addition to p-values, mean differences with 95% confidence intervals were calculated to provide effect size estimates.

RESULTS AND DISCUSSION

In *C. album*, numerical differences in the proportion of epicuticular waxes were observed, with higher values in the oldest leaves and lower in the youngest leaves (Fig. 1). In *A. theophrasti*, mean wax content tended to be slightly higher

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in the younger upper leaves compared to the lower leaves (Fig. 1). Furthermore, a One-way ANOVA revealed that the proportion of waxes in leaves did not significantly depend on the stage of development or the position of the leaf on the plant in either species (*C. album*: F=0.720, P=0.598; *A. theophrasti*: F=0.963, P=0.469). Although numerical differences in wax proportions were observed among leaf positions, these were not statistically significant (ANOVA, P > 0.05). The wax content in *C. album* leaves (ranging from 0.40 to 0.31%) exceeded that in *A. theophrasti* leaves (ranging from 0.13 to 0.18%). Therefore, the apparent trends (higher values in older leaves of *C. album*, and slightly higher values in upper leaves of *A. theophrasti*) should be interpreted with caution as indicative tendencies rather than robust effects.

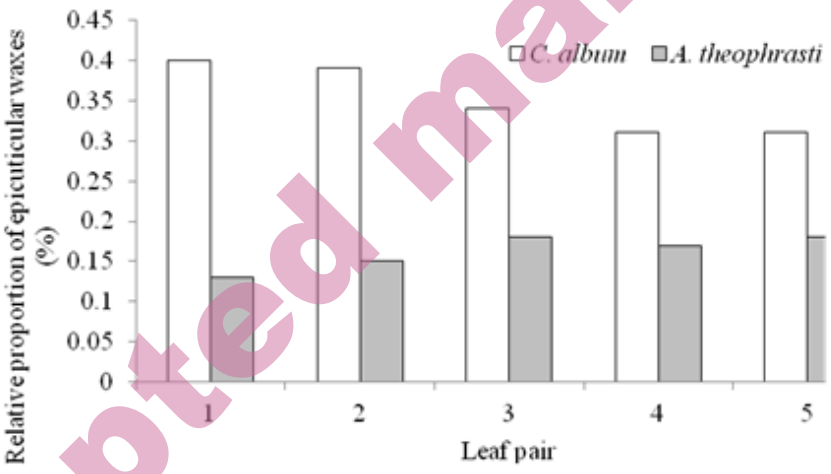

Figure 1. Relative proportion of epicuticular waxes in *C. album* and *A. theophrasti* leaves of different maturity

Analysis of the chemical composition revealed the presence of several compound classed in both species, including aldehydes, alkanes, alcohols, esters, ketones, and triterpenes. The dominant constituents in the wax composition of both species were alkanes and alcohols, with *A. theophrasti* also featuring a significant proportion of triterpenes. Additionally, *C. album* contained classes of esters and sterols, as well as ethers and sterols. In contrast, *A. theophrasti* contained fatty acids, lactones, and vitamin E. Quantitative variations in leaf epicuticular waxes were influenced by leaf age. The content of compound classes (Fig. 2) varied across different leaf stages. Specifically, the comprehensive compound list with retention times, retention indices, and identification details is provided in Supplementary Table SI and SII. In *C. album*, the content of wax components (esters, esters and sterols, ethers, ketones) was significantly higher (P<0.05) in the oldest leaves (1st pair), while sterols and triterpenes were significantly more

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abundant (P<0.05)in the youngest leaves (5th pair). Contrary, the content of aldehydes, alkanes, alkenes, and alcohols did not differ significantly among leafpositions (P>0.05), indicating relative stability of these constituents across development. In the case of *A. theophrasti*, only aldehydes and esters showed lower levels in the oldest leaves (P < 0.05), while triterpenes were significantly more abundant in the youngest leaves. Other classes (alkanes, alcohols, ketones, fatty acids, lactones, sterols, vitamin E) remained statistically unchanged (P>0.05) across leaf positions, despite minor numerical fluctuations.

As indicated, in the epicuticular wax composition of leaves of both species, alkanes and alcohols were the most abundant compounds. Regarding the content of alkanes, the chemical compounds heptacosane, nonacosane, and hentriacontane constituted the predominant share of the total wax content in leaves of *C. album* and *A. theophrasti* (as shown in Supplementary Table SI and SII). The content of these compounds varied across leaves of different growth stages or positions on the plant. Specifically, in *C. album*, the heptacosane, nonacosane, and hentriacontane content was higher in the younger leaf pair (4th or 5th pair) compared to older leaves (1st pair). Specifically, in *C. album*, the content of these mentioned alkanes fell within the following ranges: heptacosane (1.91–5.42%), nonacosane (13.34–28.37%), and hentriacontane (2.42–6.10%). In the case of *A. theophrasti*, the content of nonacosane was the most dominant component and was quantity lower in the youngest leaf (5th leaf) compared to the older leaves. Heptacosane and hentriacontane were also present in quantity higher than other components while having no such clear dependence of quantity with the age of leaves as was in *C. album* plants. For leaves of *A. theophrasti*, the measured values decreased from the oldest to the youngest leaf (heptacosane: 5.19–3.70%, nonacosane: 21.07–10.86%, and hetriacontane: 5.47–4.71%).

Within the class of alcohol compounds, octacosanol and triacontanol constituted the predominant share of total wax content in the leaves of both *C. album* and *A. theophrasti*. In *C. album*, the octacosanol content was lower in the oldest leaves (1st pair) and the youngest leaves (5th pair) compared to the other leaves. Similarly, the triacontanol content was lower in the oldest leaves (1st pair) compared to the remaining leaves. As we moved from the oldest to the youngest leaf in *C. album*, the content of these alcohols increased (octacosanol: 21.82–23.60%, triacontanol: 2.08–3.34%). For *A. theophrasti*, the content of octacosanol was lower in the oldest leaf compared to the younger (4th and 5th leaves). Additionally, a difference in triacontanol content was observed only between the oldest and youngest leaves, with higher content in the oldest leaf. The content of octacosanol compounds in leaves of *A. theophrasti* increased (5.29–7.84%), while triacontanol content decreases (2.55–1.04%).

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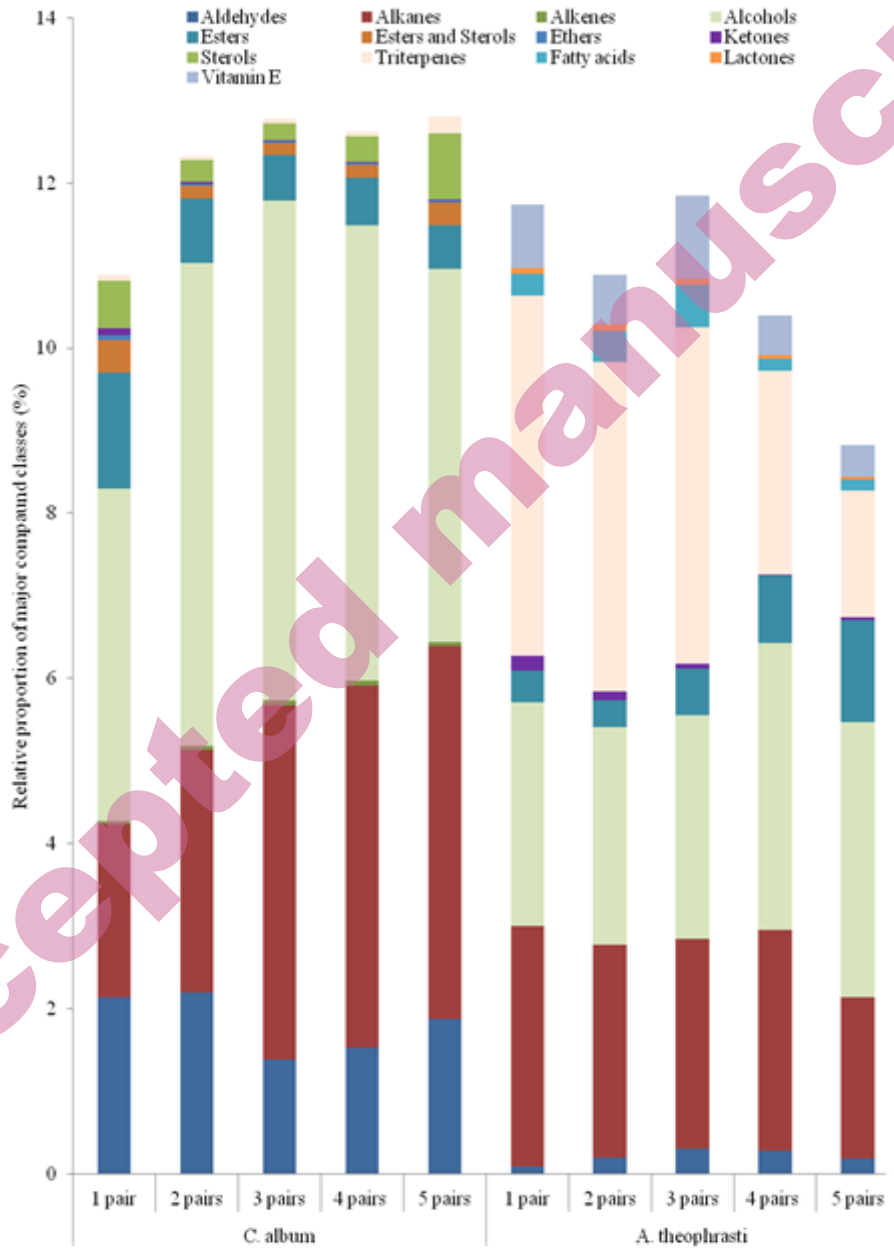

Figure 2. Major compound classes in composition of epicuticular waxes

In *A. theophrasti*, the presence of triterpenes in leaf waxes was noteworthy. Specifically, the compounds lup-20(29)-en-3-one, olean-12-en-3-one, and

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squalene dominated this class. The content of these compounds exhibited variations across different leaves. The content of lup-20(29)-en-3-one and squalene was lower in the youngest leaf (5th leaf) compared to the older leaves. Conversely, olean-12-en-3-one content was smaller in the 1st leaf compared to the other leaves. Specifically, the content of lup-20(29)-en-3-one decreased from 5.90 to 1.19%, squalene content decreases from 9.69 to 2.15%, olean-12-en-3-one content increased from 1.67 to 2.61%.

Given the distinctiveness of leaf waxes across various plant species, evaluating their chemical composition and spatial distribution within and on the leaf surface hold significant importance for chemotaxonomy.[9,10,11] Numerous studies have consistently demonstrated that the content and thickness of epicuticular waxes in leaves undergo changes as the leaves age. An analysis of wax content in *S. halepense*, revealed that the amount of wax per unit area was higher in younger plants at the 3-leaf stage (123 μg cm^{-2}) compared to older plants at the 6–7 leaf stage (38 μg cm^{-2}).[16] Several hypotheses might explained such phenomenon: younger cells might exhibited greater efficiency in wax production, or production levels remained constant regardless of plant age, but the overall wax amount decreased due to the expansion of leaf area. Our findings for the species examined in our study present a contradictory picture. Specifically, in *C. album*, the highest wax content was observed in older leaves, while in *A. theophrasti*, the younger upper leaves consistently exhibited higher wax content. Consequently, the results for *C. album* align with the perspective that wax content increases with leaf aging. Notably, older leaves tended to possess a greater proportion of epicuticular waxes, which can impede herbicide absorption and subsequent translocation to the site of action within the plant.[17]

Different plant species exhibited unique chemical profiles in their waxes. For instance, testing of *Brunnichia ovata* Walt. and *Campis radicans* L. leaves have shown that the predominant components include alkanes (24–49%), alcohols (9–61%), acids (0–11%), and triterpenes (4–62%).[18] Alkanes, primary alcohols and fatty acids were primary constituents of epicuticular wax, whereas intracuticular wax was composed of both, triterpenoids and long-chain aliphatic molecules.[19] In one research of the influence of external environmental factors on wax content in *A. theophrasti* leaves revealed similar findings.[6] Specifically, primary alcohols (29–31%), alkanes (17–20%), fatty acids (10–13%), and esters (8–11%) were identified as major constituents. Additionally, other species such as *Ipomoea hederacea* L., *Ipomoea lacunosa* L., *Ipomoea wrightii* Gray, and *Jacquemontia tamnifolia* L. exhibited varying compositions. In these species, alkanes (2958%), alcohols (19–46%), acids (5–24%), and triterpenes (0–25%) were present, with variations depending on the specific species.[17] Similarly, wax content was explored in the leaves of three poplar clones. (*Populus euramericana* cl. Pannonia (M1), *Populus deltoides* cl. PE 19/66, and cl. B229 (Bora)).[10] Notably, the highest wax

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content in poplar leaves was attributed to compounds such as nonacosane (72-78%), hexacosane (6-10%), and untriacontane (5%). In another findings it was noted that the wax content, except for alcohol in *B. ovata* and triterpenes in *C. radicans*, consistently remained higher in older leaves (5-7) compared to the youngest apical leaves (1-2).[18]

It should be noted that wax quantities were expressed as percentage of fresh leaf mass rather than per unit surface area. Because leaf water content vary strongly with age, obtained values cannot be consider as absolute wax coverage. Therefore, the data should be consider as indicator of trends in wax load and composition. Although this represents a limitation for direct conclusions about herbicide spray retention capacity, the clear developmental changes observed still provide important insight into the dynamics of wax deposition in *C. album* and *A. theophrasti* under controlled conditions. In many cultivated and woody species, including *S. halepense*, various poplar clones, and several ornamentals, wax load per unit leaf area typically declines with age, largely due to surface expansion and abrasion losses.[10,16] By contrast, our results show an opposite pattern in *C. album*, where older leaves accumulated higher proportions of epicuticular waxes. This divergence may reflect species-specific differences in cuticle thickening and prolonged activity of biosynthetic pathways in older tissues. In *A. theophrasti*, the mixed pattern (higher wax in younger leaves, attenuation in older ones) suggests a stronger influence of pubescence and trichome architecture on surface deposition and persistence of waxes.

Previous research on *A. theophrasti* indicated that environmental conditions such as light intensity and water stress change wax deposition and consequently herbicide retention.[6] In this research plants were grown under controlled chamber conditions, minimizing environmental variation and allowing us to isolate ontogenetic trends. However, in field conditions, interactions between developmental stage and environmental plasticity may either mask or amplify age-related trends. Thus, the generality of our findings should be interpreted with caution when extrapolated beyond controlled conditions.

Desribed differences in wax content between species and leaves at different positions have important implications for aproach of herbicide application. In *C. album*, the accumulation of wax on older leaves may decrease herbicide penetration, indicating the need for adjuvants involvement. Conversely, in *A. theophrasti*, dense trichomes combined with persistent wax loads in younger leaves suggest that spray retention is more affected by surface roughness than by cuticular resistance, indicating nozzle type and droplet size have critical importanve or effective coverage. Such knowledge can direct herbicide application practices, including the selection of surfactants or nozzle designs, to optimize deposition and uptake by these two species.

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The different wax contents observed between *C. album* (higher wax content in older leaves) and *A. theophrasti* (wax content tended to be slightly higher in the younger leaves than in older) may be partly explained by differences in leaf micromorphology and regulation of wax biosynthesis during leaf development.[20] *C. album* leaves are glabrous, enabling epicuticular waxes to accumulate on exposed surfaces with development, with relatively limited loss due to abrasion. In contrast, *A. theophrasti* leaves are densely hairy, and trichomes may alter the spatial distribution of waxes, providing a physical barrier that reduces direct deposition on the epidermal surface and potentially accelerates abrasion or redistribution of wax layers in older leaves. Also, different wax conten could be consecvence of different regulation of very long chain fatty acid biosynthesis genes, which drive wax production. In *A. theophrasti*, long-term activity of these pathways in developing leaves could maintain high amounts of wax in younger tissues, while older leaves partially lose it due to environmental exposure. Such ontogenetic trends are consistent with general concepts of wax biosynthesis and development.[19]

CONCLUSION

Our controlled-environment study demonstrates species-specific patterns of epicuticular wax accumulation with leaf age. In *C. album*, older leaves tended to contain higher proportions of certain wax constituents, whereas in *A. theophrasti*, younger pubescent leaves maintained relatively high wax loads. These statistically supported trends suggest that cuticle development and surface architecture interact differently across species. Importantly, such age- and species-dependent profiles have direct implications for foliar herbicide performance. In *C. album*, increased wax in older leaves may reduce herbicide penetration, requiring optimized surfactant or adjuvant use at later growth stages. In *A. theophrasti*, persistent waxiness of young trichome-rich leaves may already hinder spray retention, highlighting the need for tailored nozzle selection and spray formulations. Integrating wax metrics into herbicide application frameworks could therefore improve the effectiveness of weed management strategies for these problematic species.

Although we observed numerical variation among leaf positions, most differences were small and not statistically significant. Reported values are expressed relative to fresh leaf mass rather than per unit surface area, which limits direct inference about spray retention capacity. Therefore, our results should be interpreted primarily as reflecting relative ontogenetic trends in wax load and composition.

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SUPPLEMENTARY MATERIAL

Additional data are available electronically at the pages of journal website: https://www.shd-pub.org.rs/index.php/JSCS/article/view/13430, or from the corresponding author on request.

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ИЗВОД

ПРОМЕНЕ ЕПИКУТИКУЛАРНОГ ВОСКА КОД ЛИСТОВА *CHENOPODIUM ALBUM* L. И *ABUTILON THEOPHRASTI* MEDIK. РАЗЛИЧИТЕ СТАРОСТИ

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Епикутикуларни воскови се састоје од комплексне мешавине разлишитих органских једињења. Преовлађујућа класа једињења се састоји од *n*-алкана. *Chenopodium album* и *Abutilon theophrasti* су космополитске коровске врсте, обе од економског значаја због потешкоћа у сузбијању и обе врсте синтетишу восак на површини листова.Ово истраживање је показало да је код *C. album* садржај воскова највећи код најстаријих листова, а најмањи код најмлађих листова. Насупрот томе, код *A. theophrasti* је садржај воскова константан код млађих листова као и код старијих. Удео воскова у листовима не зависи од старости листова код обе врсте. У садржају епикутикалних воскова листова *C. album* доминирају алкани и алкохоли. Док код листова *A. theophrasti* доминирају алкани, алкохоли и тритерпени. Квантитативне варијабилности епикутикуларних воскова зависе од старости листова.

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