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CHEMICAL ANALYSIS OF LEAF CUTICULAR WAX OF POPLAR CLONES IN SERBIA

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Abstract: The leaf cuticular waxes of 3 poplar clones (*Populus euramericana*-Pannonia (M1), and *Populus deltoides* PE 19/66 and B229 (Bora)) were characterized by gas chromatography–mass spectrometry method. Poplar clones grown under identical environmental conditions showed almost identical chemical content of organic compounds within analyzed leaf cuticular wax. The dominant compound was nonacosane, with range from 72,61% ± 0,02 quantified in Pannonia clone to 78,40% ± 0,35 in B229 clone, in total cuticular wax content. Other identified compounds were hexacosane, untriacontane, octacosane, tetradecanal and triacontane; the last, triacontane, was present in very small percentage in wax content, around 1% in all three clones.

Key words: *alkanes, cuticular wax, GC/MS, leaves, poplar clones*

HEMIJSKA ANALIZA POVRŠINSKOG VOSKA SA LIŠĆA KLONOVA TOPOLA IZ SRBIJE

Površinski voskovi sa lišća od 3 klona topola (Populus euramericana cl. Pannonia (M1) i Populus deltoides cl. PE 19/66 i cl. B229 (Bora)) su analizirani metodom gasno-masene hromatografije. Klonovi topola su uzgajani pod istim uslovima spoljašnje sredine i pokazali gotovo identičan hemijski sastav u okviru analiziranog površinskog voska sa lišća. Dominantno jedinjenje je nonakozan, sa opsegom od 72,61 % ± 0,02 u klonu Pannonia do 78,40 % ± 0,35 u B229 klonu, u odnosu na ukupni hemijski sastav površinskog voska. Druga identifikovana jedinjenja su heksadekan, hentriakontan, oktadekan, tetradekanal i triakontan i poslednji, triakontan je bio prisutan u veoma malom procentu u ukupnom sadržaju površinskog voska, oko 1 % u sva tri klona.

Ključne reči: *alkani, površinski vosak, lišće, GC/MS, klonovi topola*

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INTRODUCTION

The cuticle covers the aerial portions of land plants. It consists of amorphous intracuticular wax embedded in cutin polymer and epicuticular wax crystalloids that coat the outer plant surface and impart a whitish appearance. Wax biosynthesis begins with fatty acid synthesis in the plastid. The cuticle forms a protective barrier over the aerial surfaces of plants and functions primarily as a barrier to water vapor loss (Riederer and Schreiber, 1995; Schreiber et al., 1996). Cuticular wax is hydrophobic and comprised of multiple, homologous series of long-chained lipid molecules, principally hydrocarbons, alcohols, fatty acids, sterols, ketones, and aldehydes (Bianchi, 1995; Jeffree, 1996). The chemical characteristics of the cuticular wax and increases in wax load are the primary determinants of the permeability of the plant cuticle (Schonherr, 1976; Schreiber et al., 1996). For instance, the increase in wax load has been inversely correlated with rates of cuticular transpiration in sorghum (Jordan et al., 1984) and the permeability of water and organic acids across isolated plant cuticles varies by species (Niederl et al., 1998). Not only is a plant genetically predisposed to produce this waxy cuticle, but plants can also deposit additional cuticular wax under specific environmental conditions (Giese, 1975; Blum et al., 1991; Ashraf and Idrees, 1993; Cameron et al., 2002).

Plant surface lipids are extremely diverse. They comprise alicyclic and long-chain aliphatic compounds. The common lipid classes are hydrocarbons (C21–C35), wax esters (C34–C62), ketones (C23– C33), alcohols (C22–C33), and fatty acids (C16–C32). Less common lipids, such as hydroxyl ketones, methyl and ethyl esters of fatty acids and benzoic acid, estolides, and other compounds, have also been identified (Kollatukudy, 1976; Bianci, 1995). The composition of epicuticular lipid mixture varies depending on the developmental and seasonal changes in plant. Also, morphology and composition of lipids from different parts of the same plant can be significantly different. Some studies revealed the effects of environment on plant waxes composition (Dubis et al., 1999).

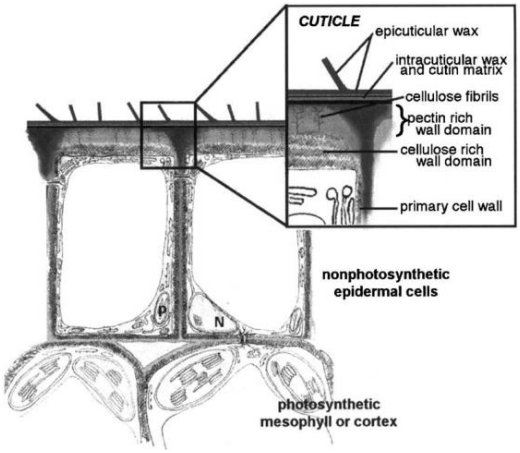
As an increase of cuticular wax synthesis during water deprivation was reported in several plants such as tree tobacco (*Nicotiana glauca* L. Graham) or sesame (*Sesamum indicum* L.), an active role of cuticle in preventing plant desiccation has been proposed (Cameron et al., 2006; Kim et al., 2007).

Besides their primary role in stress response, cuticular waxes were also found to be involved in developmental processes, notably through tight connections with the epidermis morphology (Javelle et al., 2011). The epidermal cells form a protective tissue massively dedicated to the production and secretion of the cuticle that will in turn form a continuous layer covering the epidermis. Therefore, changes in wax metabolism and transport are often associated with morphological impairment of the epidermis, most easily noticeable on specialized cells such as trichomes and stomata (Bernard and Joubes, 2013).

Washing of the foliar surface with low polarity solvents removes both the intracuticular and epicuticular waxes. The product obtained is then referred to as cuticular wax (Jetter et al., 2006). The amount of cuticular wax varies widely among plant species. For example, leaves of varieties of soybean contain 8 mg per cm^2 (Kim et al., 2007), while leaves of wild plants often contain thick deposits of cuticular wax; examples are leaves of *Tocoyena formosa* (82 mg per cm^2) and *Ziziphus joazeiro* (72 mg cm^2), both species native to Brazil (Oliveira and Salatino, 2000).

Other common wax constituents are triterpenes, while flavonoids occur more rarely in cuticular wax (Hamilton, 2004; Gao et al., 2012). Waxes play several roles in the plant biology, such as maintenance of an impermeable foliar surface (and thus contributing to avoid the growth of pathogens), restriction of the loss of water and protection against the attack of herbivore insects and UV irradiation (Baker, 1982; Hamilton, 2004; Gao et al., 2012; Kitagami et al., 2013).

Consistent with its major role in plant/environment interactions, wax synthesis was shown to be under environmental regulation. A transcriptional regulation of numerous wax-associated genes was elucidated by gene expression monitoring during differential environmental conditions (Bernard and Joubes, 2013)



Picture 1. Generic representation of transverse views of wax secreting epidermal cells, showing the components of the cuticle, cell wall domains, and the nonphotosynthetic epidermal cell (taken from Kunst and Samuels, 2003)

Slika 1 . *Generički prikaz poprečnog preseka ćelija epiderma lista koje luče vosak, pokazujući komponente kutikule, domene ćelijskog zida i nefotosintetičkih epidermalnih ćelija (preuzeto iz Kunst i Samuels, 2003)*

The aim of study

There is no data on the cuticular wax composition of any plant from *Salicaceae* family in Serbia. As the objective of the study was to examine the wax composition and not the variability between single plants, quantitative results were obtained for one pooled sample of leaves from five plants per cultivar.

MATERIALS AND METHODS

Poplar plant material was sampled on 7th of August, 2013 in early morning hours. It was taken 4-8 leaves from five 2 year old seedlings from all three clones, provided from the Institute of Lowland Forestry and Environment (Novi Sad, Serbia) collection. The intact leaf samples were imidiately transported in sterile plastic bottles (5 ml volumes) on ice to laboratories of Centre for Instrumental Analysis in Belgrade. Washing of cuticular waxes for every sample was done with aproximately 10 ml analyticaly pure destilated n-hexan in sterile glass erlenmeyer and the extracts were evaporated under a stream of N_2 and the dried wax residues were prepared for further GC-MS analysis.

The quantification of the compounds was based on their peak areas from the GC-FID analysis, compared to the peak areas of the internal standards (nonacosane, hexacosane, untriacontane, octacosane, tetradecanal and triacontane). All standards (purity 98–99%) were obtained from Sigma-Aldrich. Determination of exact chemical content of leaf cuticular wax was done on samples taken from three poplar clones, belonging to species: *Populus euramericana cl*. Pannonia, *Populus deltoides cl*. PE 19/66 and *cl*. B229, grown in the same environemntal conditions in Kać Forest estate, near Novi Sad city.

GC/MS and GC-FID analyses

GC/MS and GC-FID analyses were carried out with an Agilent 7890A apparatus equipped with an auto-injection system (Agilent GC Sampler 80), an inert 5975C XL EI/CI mass-selective detector (MSD) and a flame ionization detector (FID) connected by a capillary flow technology 2-way splitter with make-up, and a HP-5 MS fused-silica cap. column (30 m_0.25 mm i.d., film thickness 0.25 mm). The oven temperature was programmed linearly rising from 60 ^{0}C to 3008 ^{0}C at 38/min and then isothermal at 3008 ^{0}C for 10 min; injector temp., 2508 ^{0}C; detector temp., 3008 ^{0}C; source temp., 2308 ^{0}C; quadrupole temp., 1508 ^{0}C; carrier gas, He (16.255 psi, constant pressure mode). Samples (1 ml) were injected in splitless mode. Electron-impact mass spectra (EI-MS; 70 eV) were acquired over the m/z range 30–550. The solvent delay was 3 min.

The components were identified based on the comparison of their retention indexes (RIs) with those of reference spectra (Wiley and NIST databases) as well as

by the retention time locking (RTL) method and comparison with the RTL Adams database. The RIs were experimentally determined using the standard method described by Van Den Dool and Kratz, (1963) i.e., they were established related to the retention time (tR) of n-alkanes injected after the sample under the same chromatographic conditions. The relative abundance of the n-alkanes was calculated from the signal intensities of the homologues in the GC-FID traces.

The identification of the compounds was based on comparison of their EI mass spectra with the NIST MS Search 2.0 computerized mass spectral libraries Wiley7 and Nist05, and with the available literature EI-MS data of the compounds previously identified in the defensive secretions of millipedes (Attygalle et al., 1993). All chemical analysis was performed in Centre for Instrumental Analysis, Faculty for Chemistry, University of Belgrade.

Picture 2. GC-MS Agilent system used for identification of evaporative chemical compounds from poplar leaves (picture taken by the first author, on 16th of August, 2013 in main Centre for Instrumental Analysis laboratory, Faculty for chemistry in Belgrade)

Slika 2. *GC-MS Agilent sistem koji se koristi za identifikaciju isparljivih hemijskih jedinjenja iz lišća topola (sliku kreirao prvi autor, 16. avgusta 2013., u laboratoriji Centra za instrumentalnu analizu, Hemijskog fakulteta u Beogradu)*

Statistical analysis

Identification and quantifiation of compounds from cuticular wax was done through GC/MS spectres analysis in GCMS Data Analysis and AMDIS 31 programmes. Measurement error for five samples per clone was calculated as ± one standard deviation in Excel Windows.

RESULTS AND DISCUSSION

Table 1. Results for the GC/MS analysis of cuticle wax of three poplar clones
Tabela 1. *Rezultati za GC/MS analize kutikulularnog voska tri klona topola*

The name of compound *Ime jedinjenja*	Retention time *Retenciono vreme*	Retention index *Retencioni indeks*	Partition in total wax *Učešće u ukupnom vosku*		
			Pannonia	B229	PE 19/66
Nonacosane	72.043	2894.3	72,61% ± 0,02	78,40% ± 0,35	75,28% ±0,044
Hexacosane	67.053	2681.4	10,88% ± 0,009	6,49% ± 0,009	7,84% ± 0,016
Untriacontane	76.418	3097.3	5,87% ± 0,005	5,82% ± 0,016	5,34% ± 0,004
Octacosane	69.512	2788.3	4,51% ± 0,012	3,06% ± 0,007	3,03% ± 0,0044
Tetradecanal	79.551	3242.1	2,40% ± 0,002	3,38% ± 0,011	3,85% ± 0,0088
Triacontane	74.173	2993.1	1,74% ± 0,0013	1,43% ± 0,001	1,24% ± 0,002

Research on n-alkanes has most frequently been used in chemotaxonomic studies of trees and herbaceous plants. n-Alkanes in plants, in combination with other chemical markers, are also valuable for analyses in other fields, i.e., phylogenetic studies, hybrid detection, air pollution studies, nutrition studies etc. They can be used as chemotaxonomic markers at the generic level, in environmental studies, in paleo-environmental reconstructions etc. The n-alkanes in conifers have been most extensively studied in *Picea* and *Pinus* genera (Bojović et al., 2012)

Hydrocarbons with 16–37 carbon atoms were detected in *Solanum macrocarpon* leaf waxes, compounds n-C31 and n-C33 alone making up more than 60% of the total alkanes. (Helinski et al., 2012). In this regard, the predominance of alkanes over other classes of wax constituents may be an additional factor accounting for the hardiness by *Coffea racemosa*. Among the common classes of foliar wax components, alkanes may be the most efficient as a barrier to cuticular transpiration (Oliveira et al., 2003). The distributions of n-alkanes and n-primary alcohols have been used to characterize and distinguish among species and varieties of *Coffea* (Stocker and Wanner, 1977; Kitagami et al., 2013).

In the table 1. are presented results of the GC/MS analysis of cuticle wax of three poplar clones from Serbia. n-Nonacosane is the most prominent alkane with long chain, with values from 72,61% ± 0,02 for Pannonia clone and 78,40% ± 0,35 for PE 19/66 clone. The next one is n-hexacosane, where clone Pannonia showed the biggest content in cuticular wax with the procentage of 10,88% ± 0,009. B229 and PE 19/66 showed less content, 6,49% ± 0,009 for B229 and 7,84% ± 0,016 for PE 19/66. n-Untriacontane is present in all three clone with around 5% in total wax content. n-Octacosane is most present in Pannonia clone (4,51% ± 0,012**),** while n-tetradecanal and n-tracontane are present in traces (between 1% and 3% in total wax content)**.** n-Heptacosane (C27), n-nonacosane (C29), and/or n-hentriacontane (C31) are commonly the most dominant members of the n-alkane homologues in cuticular wax (Gulz, 1994). In the paper of Cameron et al., (2012) n-heptacosane was the major alkane component for the *Salix* clones, whereas n-nonacosane was the major alkane component for the hybrid poplar clones representing 17–33% of the total wax

load. Comparing to our results, the similarity is within presence of n-nonacosane as the most abundant compound, although in our clones it was identified in approximately 70% of total amount of alkane content. In our samples, n-hexacosane was next most abundant alkane which is between n-pentacosane and n-heptacosane, indicating similar biosynthetic pathway. The relative abundance of n-heptacosane in *S*. *eriocephala* wax ranged from 3–5% of total wax load, compared to *S*. *dasyclados* where the proportion of n-heptacosane was close to 27% for all three sampling periods. n-Pentacosane and n-hentriacontane represented only a small portion of the total wax load. The pattern of deposition was also different between the *Populus* species hybrids and the *Salix* species. The *Populus* species hybrids displayed a much smaller proportion of both n-heptacosane and n-nonacosane in the September sampling compared to the May sampling, whereas both *S*. *purpurea* and *S*. *dasyclados* had a greater relative abundance of n-nonacosane in the September sampling compared to the May sampling. Systematic studies of the large collection of diverse wax mutants now available should highlight the specific contribution of single wax compounds in plant/environment interactions as well as in the organization of waxes, together with cutin, in the highly structured cuticle (Bernard and Joubes, 2013).

CONCLUSION

In this work we report for the first time the chemical composition of the cuticular waxes of poplar clones from Serbia.

Our results showed that there are no significant differences in presence in organic compound of leaf wax. Quantitative differences between clones are small and bigger numbers of samples are needed to determine exact population differences among clones related to identified compounds. It will be recommendable to do comparison in wax compound analysis between clones from estate and grown in plant tissue culture also. In that way, we may determine how much environmental conditions are influencing on synthesis of cuticular wax and its content and afterwards, apply abiotic stress, like simulated drought or increased heavy metals concentration in culture medium.

The full characterization of the usefulness of poplar clones plants in agriculture of developing countries should cover also the analysis the wax components during different abiotic stresses, with emphasize on climate changes. Further studies are needed to assess the content of such compounds in both poplar and willow species from Serbia, to clear which factors may influence on cuticle wax content and may it be used as chemotaxonomic intra-and interspecies marker system.

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R e z i m e

HEMIJSKA ANALIZA POVRŠINSKOG VOSKA SA LIŠĆA KLONOVA TOPOLA IZ SRBIJE

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Površinski vosak na lišću viših biljaka je hidrofobni sloj koji se sastoji od niza dugolančanih lipidnih molekula i alkoholnih jedinjenja. Biljni površinski lipidi su vrlo raznoliki. Oni obuhvataju alicikličkа i dugolančana alifatska jedinjenja. Zajedničke klase lipida su ugljovodonici (C21-C35), voštani estri (C34-C62), ketoni (C23-C33), alkoholi (C22-C33), i masne kiseline (C16-C32). Hemijske karakteristike voska i povećanje njegovog površinskog napona su primarne determinante propustljivosti biljne lisne kutikule. Osim svoje primarne uloge u odgovoru na stres, površinski voskovi su takođe uključeni u razvojne procese morfologije lista, naročito kroz njegove uske veze sa tkivom epidermisa.

Nema podataka o hemijskoj analizi sastava površinskih voskova bilo biljke iz porodice Salicaceae u Srbiji. Površinski voskovi sa lišća od 3 klona topola (Populus euramericana -Pannonia (M1) i Populus deltoides PE 19/66 i B229 (Bora)) su analizirani metodom gasno-masene hromatografije. Klonovi topola su uzgajani pod istim uslovima spoljašnje sredine i pokazali gotovo identičan hemijski sastav u okviru analiziranog površinskog voska sa lišća. Kvantitativni rezultati dobijeni su za jedan grupni uzorak lišća od pet biljaka po klonu. U tabeli 1. su predstavljeni rezultati GC/MS analize kutikule voska tri klona topola iz Srbije. n-nonakozan je najzastupljniji alkan dugog lanca, sa vrednostima od 72,61% ± 0,02 za klon Pannonia i 78,40% ± 0,35 za PE 19/66 klon. Sledeći je n-heksakozan, gde je takođe za klon Pannonia registrovan najveći sadržaj u ispitivanom površnskom vosku od 10,88% ± 0,009. B229 i PE 19/66 su pokazali manje sadržaja, 6,49% ± 0,009 za B229 i 7,84% ± 0.016 za PE 19/66. n-triakontan je prisutan u sva tri klona sa oko 5% u ukupnom udelu voska. n-oktakozan je kvantitativno najviše prisutan u Panonnia klonu (4,51 ± 0,012%), dok su n-tetradekanal i n-triakontan prisutni u tragovima (između 1% i 3% u ukupnom sadržaju voska).

Naši rezultati su pokazali da nema značajne razlike u prisutnosti u hemijskom sastavu površinskog voska lista sa uzorkovanih klonova. Kvantitativne razlike između klonova su mali i veći broj uzoraka je potreban kako bi se utvrdila preciznija razlika između klonova u odnosu na identifikovana jedinjenja. Isto tako, uporedna analiza hemijskog sastava površinskog voska između uzorkovanih klonova iz spoljašnje sredine i kulture tkiva bi predstavljao sledeći korak ka identifikovanju tačne uloge voska na metabolizam stresa kod naših biljaka i izloženim različitim tipovima abiotskih stresova.