PERIODICUM BIOLOGORUM
VOL. 125, No 1–2, 27–34, 2023
DOI: 10.18054/pb.v125i1-2.24852

UDC 57:61
CODEN PDBIAD
ISSN 0031-5362

Original Research Article

Morphology of extrafloral nectaries of *Ailanthus altissima* (Mill.) Swingle (Simaroubaceae)

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Keywords: excretion; invasive alien plant species; light microscopy; SEM; tree of heaven

Abbreviations
EN – extrafloral nectaries
SEM – scanning electron microscopy

Received January 3, 2023
Revised February 9, 2023
Accepted February 15, 2023

Abstract

Background and Purpose: Ailanthus altissima *(Mill.) Swingle (Tree of Heaven) is a highly invasive, widespread, and widely investigated plant species native to the broader area of China. Extrafloral nectaries are major components of the* A. altissima *secretory system, but the knowledge of their morphology and role in the tree's physiology is limited. This research aims to explore the morphology of extrafloral nectaries, compare it to previous findings, and discuss their possible role and function.*

Materials and Methods: *Extrafloral nectaries on leaves have been monitored through different phases of leaf development, from June to August 2015 and 2022. The nectaries' morphology was investigated using Zeiss Axioscope 5 and BOECO BSZ-405 light microscopes, and FEG QUANTA 250 FEI scanning electron microscope, operating at 7 kV and pressure of 60–100 Pa, without samples pretreatment.*

Results: *Our investigation revealed the absence of earlier reported pores or ducts on the top of the glands. However, it supports one of the first, systematical investigations of* A. altissima *extrafloral nectaries conducted in Croatia a century ago, which was until recently forgotten by history. We evaluated our findings against prior theories and assessed the nectaries' potential role and purpose in disposing surplus sugars.*

Conclusions: *The nectar in* A. altissima *is not secreted via an opening on the leaves but rather through epidermal tissue tearing.*

INTRODUCTION

Ailanthus altissima (Mill.) Swingle, or Tree of Heaven, is a deciduous tree of the Simaroubaceae family. It is a highly invasive plant, included in the Global Register of Introduced and Invasive Species *(1)* and the List of invasive alien species of concern in the EU *(2)*. Native to the broader area of China, it is now widespread on all continents except Antarctica *(3)*. The species is most abundant in urban areas, along transport corridors, or other disturbed sites, such as agricultural fields or construction sites, where it damages infrastructure and affects human health *(4)*. It can also invade natural habitats where it overthrows native species (Figure 1A, B). However, this species also provides a range of ecosystem services, such as medicinal, reducing environmental pollution, phytoremediation, land reclamation and erosion control *(5)*. Allelopathic and medicinal effects of *A. altissima* are widely investigated and reviewed *(4,6,7)*. One of the old names for the species, *Ailanthus glandulosa* Desf. *(8)*, referred to the pronounced leaf secretory glands – extrafloral nectaries (ENs).

Figure 1. Ailanthus altissma *(A) tree, (B) foliage.*

Extrafloral nectaries (ENs) are specialized nectar-secreting plant glands that develop outside of flowers and are not involved in pollination *(9)*. They have been noted on a highly diverse range of species, encompassing more than 100 families and 745 genera *(10)*, showing considerable diversity in evolutionary origins and morphology *(11)*. ENs are most often associated with defensive mutualisms, providing a nutrient source to animal mutualists, which in turn provide plant protection against herbivores *(12)*. Except for the ecological function, we know very little about the function of ENs in plants' physiology. One of the hypotheses is that the role of ENs is to excrete metabolic waste *(13)*. The position of ENs in plants is common in leaves, petioles, stipules, young stems, flower pedicels or external floral organs that do not participate in pollination *(9,11)*. Five structural types – flattened, raised, pit, scale-like, and formless nectaries – were also ascribed to ENs *(14)*.

Despite the morphology of *A. altissima* being laboriously researched and described by Kowarik and Samuel *(6)*, the investigation into the morphology and physiology of the species' ENs is not particularly extensive *(15,16)*. The nectaries' presumed function is to eliminate excess sugar from the plant. Both the floral and extrafloral nec-

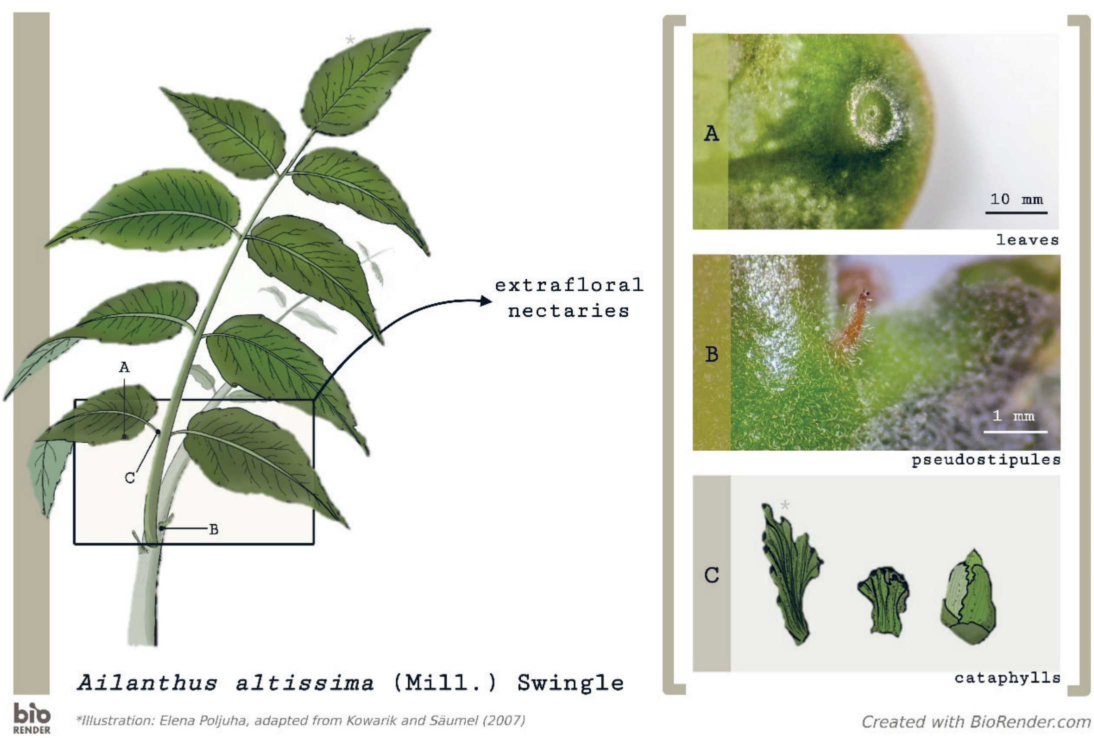

Figure 2. *Extrafloral nectaries in* Ailanthus altissima *developed on (A) leaves, (B) pseudostipules, and (C) cataphylls.*

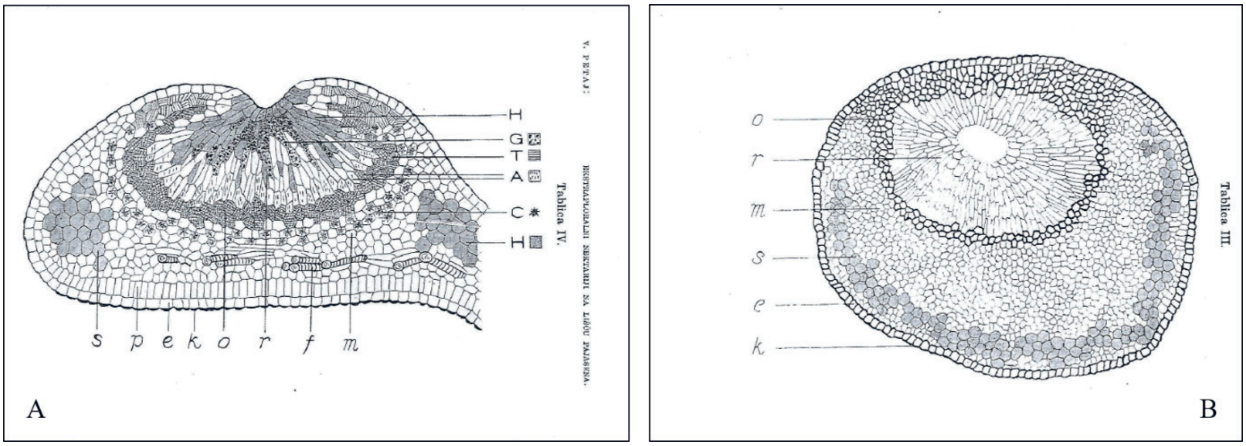

Figure 3. *Hand-drawn cross-sections through EN of* A. altissima. *(A) The radial cross-section through EN on a mature leaf, showing four separate histochemical reactions for proving the nectar composition; (B) The transverse cross-section through EN on a young leaf, stained by Flemming's triple staining method (Safranin-Gentian Violet-Orange G). A – starch in peripheral EN cells, C – calcium oxalate, e – epidermis, f – vein, G – nectar, H – resin, k – cuticle, m – mesophyll, o – belt, p – palisade, r – radially elongated cells, s – secretory cells, T – tannins. Images taken from: Petaj V. Ekstrafloralni nektariji na lišću pajasena (*Ailanthus glandulosa *Desf.); https://dizbi.hazu.hr/a/?pr=i&id=170434 © Hrvatska akademija znanosti i umjetnosti, DiZbi. HAZU, 2018.*

taries of *A. altissima* are found to contribute to this process, excreting different sugar types *(17)*. Further research into the ENs was focused on their function regarding the context of myrmecophily and their role in the ecological relationships of the plant species *(18)*.

The extrafloral nectaries in *A. altissima* are situated at the tip of the two to four teeth on the margins of the leaflets on the abaxial surface of young and mature leaves. They can be found also on the top of the structures known as pseudostipules, and on the margins of cataphylls (Figure 2) *(6)*.

Recent papers lack research on the ENs' morphology and their possible functions. Historically relevant but unknown findings previously attempted to elucidate their function. One of the first studies of the *A. altissima* ENs is the doctoral dissertation of the first woman who earned a PhD in botany in Croatia, Vjera Petaj, from 1916 *(19)*, which provides a detailed morphological, anatomical and histochemical description of ENs and proposes the mechanisms of nectars excretion (Figure 3A, B).

The results of this study differed from existing anatomical studies at the time (20,21) but also from more recent topic research *(15,16)*. Myrmecophilous relationship discounting the current function of the ENs was not determined in her study, as opposed to the findings of Delpino from 1886 *(22)* and Macchiati from 1899 *(23)*.

In our study, we aimed to explore the morphology of extrafloral nectaries using light and scanning electron microscopy (SEM) techniques. We also compared our results with earlier theories and discussed the possible role and function of extrafloral nectaries in the excretion and elimination of excess sugars and regulation of photosynthetic activity.

MATERIALS AND METHODS

Extrafloral nectaries on *A. altissima* leaves have been monitored through different phases of leaf development – from young to old, from June to August 2015 and 2022. In total, 80 samples were collected from plants (no voucher, wild collected, Croatia) at four locations in the urban parts of the cities of Pula and Poreč in Istria (Croatia) (Table 1). We visited each location twice during each season and collected 10 samples (shoots) each time. Young reddish and slightly older leaves without the visible presence of anthocyanins, and mature, fully developed leaves from the top shoots of trees and shrubs, taken from a height of up to 2 m, were collected. Microscopic analyses were performed immediately after collecting fresh leaves.

Table 1. *Locations of* A. altissima *sampling.*

Location	Lattitude	Longitude
Poreč Žatika	45°13'24.8"N	13°36'28.9"E
Poreč Sv. Marko	45°13'16.3"N	13°36'05.4"E
Pula Lučica Delfin	44°50'44.5"N	13°50'26.9"E
Pula Valsaline	44°50'48.3"N	13°50'15.8"E

The light microscopy observations of the extrafloral nectaries were made on transverse and superficial free-hand sections using a BOECO Zoom Stereo Microscope BSZ-405 (Boeckel +Co, Hamburg, Germany) and Zeiss Axioscope 5 optical microscope (Zeiss, Oberkochen, Germany). B-view (Boeckel +Co, Hamburg, Germany) and ZEISS ZEN 3.3 (Zeiss, Oberkochen, Germany) software were used to measure the structures. Fresh samples, without pretreatment, were observed using a QUANTA 250 FEG scanning electron microscope (SEM) (FEI Europe B.V., Eindhoven, The Netherlands) operating under low vacuum conditions at 7 kV and pressure of 60–100 Pa.

RESULTS

The investigation of EN morphology in this paper aims to verify the contradictory claims of modern *(15,16)* and centennial *(19)* anatomical investigations of one of today's most invasive species, *A. altissima*. For this purpose, extrafloral nectaries on leaves have been monitored through different phases of leaf development.

The external appearance of the leaf ENs resembles hemispherical swellings with a diameter of 1–2 mm, situated at the tips of the two to four leaf teeth on the abaxial surface of mature leaves, 8–19 cm long (Figure 4C, D). They are especially prominent above the veins of young leaves 1–4 cm long (Figure 4A, B). In the early stage, when the leaves are 1–2 cm long, the glands are located on the adaxial lamina of the leaf and contain anthocyanin, which contributes to their more distinct coloration. In the later stages, the ENs lose anthocyanin and become intensely green, while the young leaves remain red (Figure 4B). The leaf twists, and the ENs from the top of the leaf and vein move to the abaxial side of the leaf. During the leaf ripening, the glands become flatter, with a depression in the center, where the nectar accumulates. The completely differentiated ENs are recognized by concentric circles that appear on the gland; the bright circle on the outside and the dark green one inside, while a dark spot becomes visible in the center (Figure 4D).

In ENs, different tissues can be distinguished: the single-layered epidermis, the nectary parenchyma, and the subnectary parenchyma, as defined by Nepim (24). The gland tissue consists of an elliptical-round complex of cells, densely packed and radially elongated towards

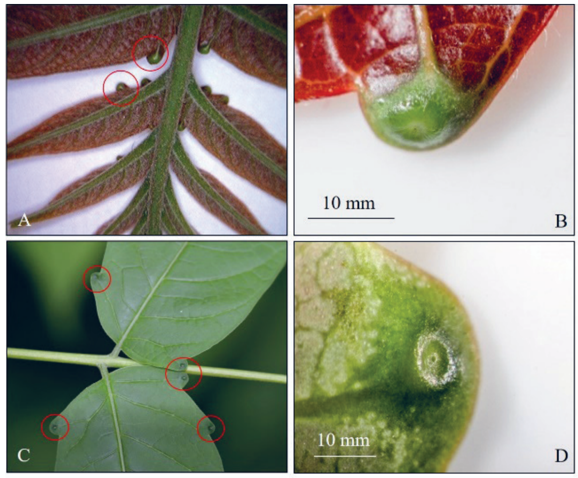

Figure 4. *Extrafloral nectaries (red circles) on the young (A, B) and mature (C, D) leaves of* A. altissima.

the center, surrounded by a band of less compact cells with chloroplasts. Under the nectary tissue is a mesophyll with characteristic isodiametric and tangentially narrowed cells and vascular bundles (Figure 5A, B).

In order to check whether there is a pore at the top of the gland, through which nectar is secreted, as described by some authors *(16,21)*, and some refute *(19)*, we investigated the EN's morphology by SEM.

Our investigation did not reveal any pores at the top of the glands. Although the nectar was found (Figure 6F), the absence of the pore could be clearly noticed (Figure 6D, E).

DISCUSSION

Compared to scarce findings on *A. altissima* gland morphology, we noticed differences in ENs structure

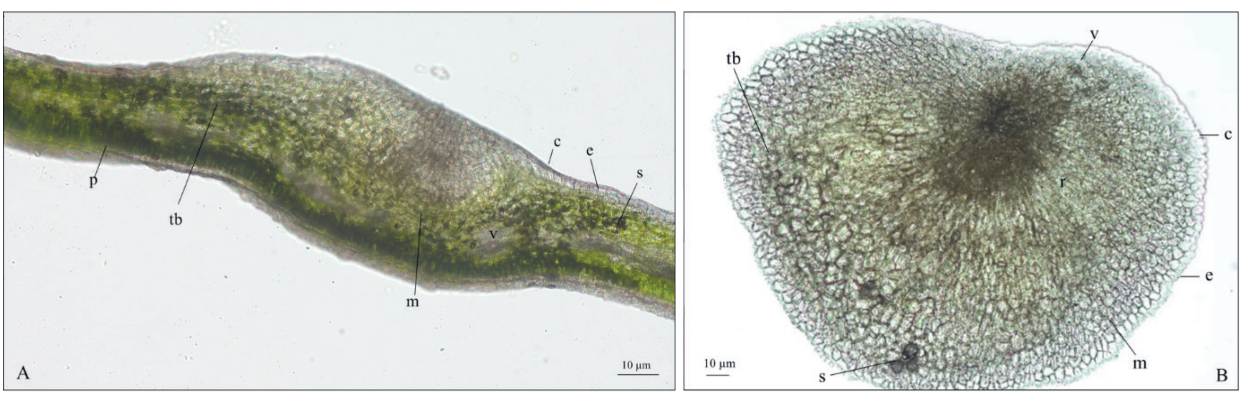

Figure 5. *Anatomy of foliar nectaries. (A) Longitudinal light-microscope section (B) Transverse light-microscope section. (e – epidermis; c – cuticle; m – mesophyll; v – vascular system; r – radially elongated cells; p – palisade parenchyma; s – secretory cells; tb – tannin-containing cells belt).*

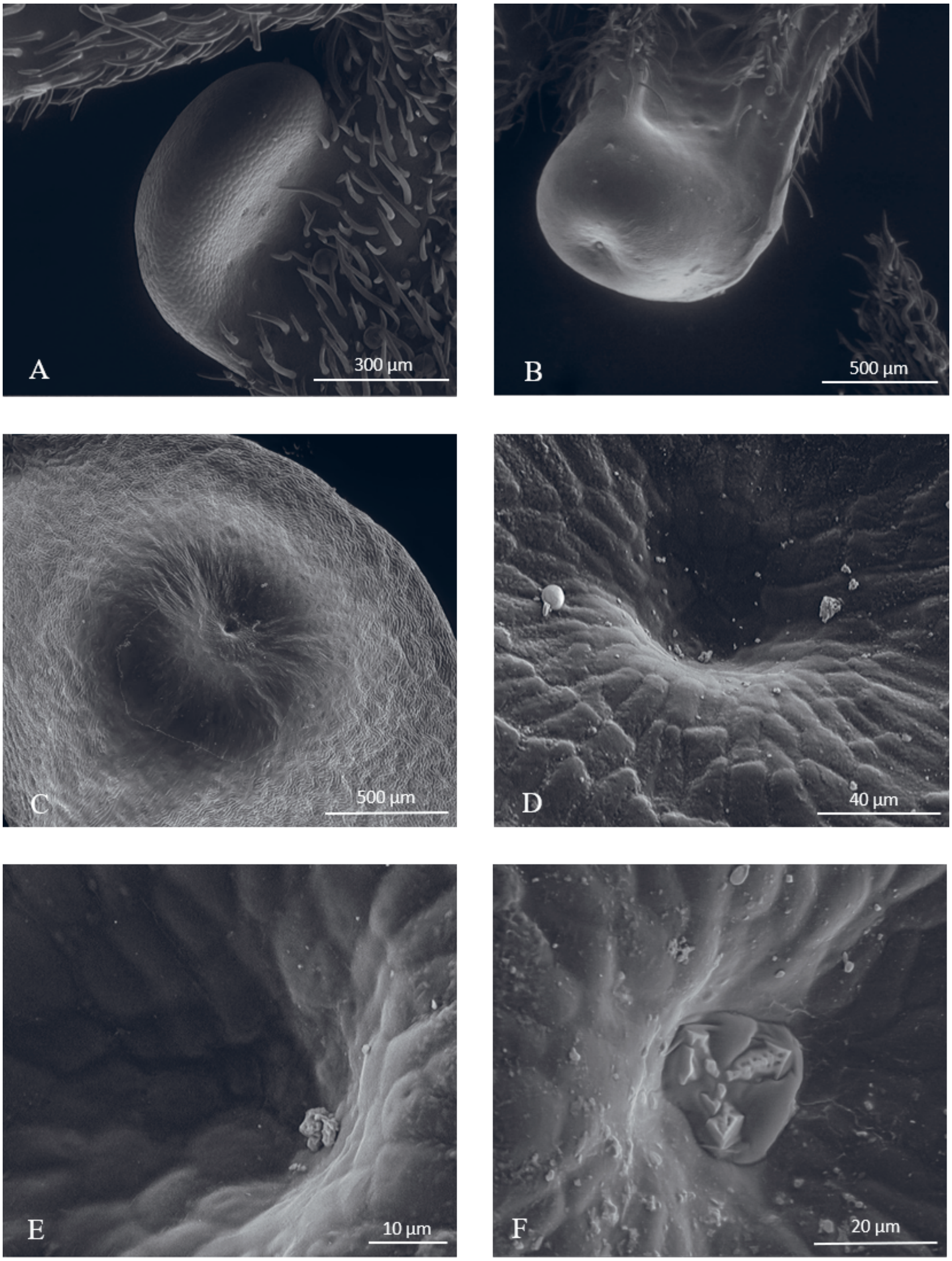

Figure 6. *Scanning electron micrograph of extrafloral nectaries in* A. altissima. *(A, B) Nectaries developing in younger leaves; (C-E) Nectaries along the basal margins of the leaflets in mature leaves; (F) crystallized nectar.*

compared to the recent literature *(15,16)*. We found crystallized nectar in the recess at the top of the gland (Figure 6F), but not the pore (Figure 6D, E) through which this nectar is secreted, as stated by other authors *(16,21)*. Although we do not exclude the presence of a pore in nectaries on cataphylls and pseudostipules, we did not observe any in the leaf nectaries. Since no excretory duct was found, we presumed that nectar was excreted directly through the epidermis. Bernardello *(25)* summarized several ways in which nectar can be excreted through epidermal cells: crossing the plasmatic membrane and the cell wall, accumulating between the cell wall and the cuticle, which breaks under the nectar pressure, or through orifices, modified stomata or small pores. In our investigation we did not reveal either stomata or orifices, so we presume that the pores observed by other authors *(16,21)* probably resulted from the rupture of the cuticle outer layer due to increased osmotic pressure caused by nectar accumulation in mature leaves. However, our results, that revealed the absence of pores or ducts, support one of the first systematic investigations of *A. altissima* extrafloral nectaries conducted by Vjera Petaj a century ago *(19)*. Her PhD research aimed to describe extrafloral nectaries of *A. altissima* which were up to then scarcely investigated. She precisely demonstrated the anatomical structure of the ENs (cf. Figure 3A, B), proved evidence for nectar generation by microchemical tests and determined the mechanism of its excretion.

In her study, Petaj corrected and supplemented the anatomical studies of Solereder (1899–1908) *(20)* and Van Tieghem (1906) *(21)*. She asserted that EN consists of a) glandular or nectary tissue, b) epidermis, c) mesophyll that surrounds the glandular tissue from all sides, d) vascular tissue, and e) excretory cells. Similar histological zones were also detected later by other authors *(16)* and in our study (Figure 5A, B). Furthermore, her investigations *(19)* revealed that there is no intercellular space in the glandular tissue, that the epidermis is not thinner in the middle of the recess and that the epidermis has no stomata. According to her observations, the vessel does not enter into the EN tissue and is not connected with secretory cells.

By microchemical analysis, she determined that nectar is produced within the nectary tissue, in the belt and its neighboring cells. She determined glucose, fructose, and sucrose, as the main ingredients of nectar and stated that nectar is formed by hydrolysis of starch in the cells on the periphery of the nectary tissue *(19)*. In addition to nectar, she proved the presence of starch, resin, tannins and diastatic ferment in the nectary tissue. She assumes that the role of tannins, formed in the early phase of EN differentiation and abundant in the cells of the belt around the glandular tissue, is to prevent the secretion of nectar into the surrounding assimilation tissue. Later research showed that the composition of nectar varies according to the type of nectary *(26)*. The nectar from the *A. altissima* leaf nectaries consists predominantly of sucrose, glucose, and a particularly high percentage of fructose content, characteristic to the species itself *(17)*. In addition, oleic, palmitic, and linoleic acids, and bound lipids (monogalactosyldiacylglycerol) have been identified in the secreted substance *(17)*.

With regard to the method of secretion, Petaj *(19)* observed that there is no special aperture or duct on the nectary, which was also confirmed by our research, but the nectar is excreted directly through the epidermis. In young nectaries, it is covered with a thin cuticle, and later, when the leaf is older, the cuticle thickens, and so does the cuticle above the nectary. Due to the osmotic pressure, both the cuticle and the neighboring nectary tissue burst, and nectar comes out through this torn tissue. However, according to her observations (19), that opening does not remain permanently, but is blocked by a subsequently formed cork cambium, and in the same way, a new opening is created in another place during a new excretion.

Our study did not extend to investigating the role of ENs in the physiology of the species. However, Bory and Clair-Maczulajtys *(16)* conducted a series of experiments to determine the function of the ENs in *A. altissima*, finding that the activity of the foliar nectaries correlates with the requirements for photosynthetic products. By removing carbohydrate-rich nectar, the plant regulates the metabolic intensity of photosynthesis, allowing it to extract metabolic products without accumulating excess starch. Nectaries' removal causes the reduction of nectar flow and starch accumulation in the leaves, resulting in a reduced rate of photosynthesis. This suggests that foliar nectaries participate in the regulation of photosynthetic activity *(16)*. Our research provides evidence-based arguments confirming Petaj's observations on the theory that the nectaries' primary role was to foster mutualistic relationships with insects.

Our research uses analytical methods revealing ENs structure and bridges the gap between earlier research, historical arguments, and contemporary observations. We conclude that the nectar in *A. altissima* is not secreted via an opening on the leaves but rather through epidermal tissue tearing, as first described by Petaj *(19)*.

Acknowledgement: *This research was supported by the Croatian Science Foundation under the project "NATURe as an ALLY: Alien Invasive Plants as Phytopharmaceuticals – NATURALLY" (IP-2020-02-6899). The work of Mirela Uzelac was supported by the Croatian Science Foundation "Young Researchers' Career Development Project–Training New Doctoral Students" (DOK-2021-02-6899). We would like to thank the Croatian Academy of Sciences and Arts HAZU for providing the images from the digital archive collection DiZBi. We also thank Elena Poljuha for the botanical illustration of A. altissima.*

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