PRELIMINARY STUDIES ON THE STRUCTURE OF SEPALS AND TRICHOMATOUS NECTARIES IN FLOWERS OF *Tilia cordata* Mill.

Agata Konarska
University of Life Sciences in Lublin

**Abstract.** *Tilia cordata* is a good source of food attractants for bees. These insects are the primary pollinators of flowers of this species. Nectar is produced in the floral trichomatous nectaries located on a projection of the adaxial, basal part of the sepals. There were two types of non-glandular hairs on the sepals; the first one to prevent the nectar from flowing out beyond the sepal and the other type to protect the nectary itself. The clavate secretory trichomes, forming dense clusters, are composed of a base, stalk, and multicellular head. The secretory cells of the trichome head contain a thick cytoplasm and a large nucleus; they are also characterized by a low degree of vacuolation. Nectar accumulates at the tip of the trichome in the space formed between the cell wall of the head cells and the cuticle. The several-layered subepidermal glandular parenchyma with densely packed cells is provided with vascular bundles containing xylem and phloem. Plastids containing small starch grains were noticed in many cells of the nectariferous tissue, whereas phenolic compounds were found in the adaxial epidermal cells of the sepals. No presence of starch, lipids, or phenolic compounds was found in the cells of the glandular hairs. However, numerous chloroplasts, calcium oxalate crystals and large mucilage cavities occurred in the subglandular tissue.

**Key words:** Malvaceae, secretory trichomes, micromorphology, anatomy, mucilage cavities, phenolic substances

**INTRODUCTION**

Beside *Tilia platyphyllos* L., *Tilia cordata* Mill. is one of two lime species found in the wild in Poland which currently belong to the family Malvaceae, subfamily Tilioideae. This tree also grows across Europe, in the Caucasus, and in Western Siberia [Szweykowska and Szweykowski 2003]. Due to its quite long flowering period (10–12 days) and abundant flower production in the inflorescence (5–20), small-leaved lime is considered to be a good source of pollen and nectar for bees [Győr 2001, Pogorzelec...
During the flowering period, bees visit lime flowers from early morning hours until late evening. According to different sources, lime nectar is characterized by a variable content of sugars; it ranges from 20 to 70% depending on the time of the day and the stage of flower development. The dominant sugar in its nectar is sucrose which accounts for ca. 19% of total sugars. [Halmágyi 1975, Czubacki 1996, Jabłoński and Kołtowski 1999]. Honey yield of *T. cordata* is from 100 up to 600 kg per ha (max. 1000 kg) [Crane et al. 1984, Pogorzelec 2006], whereas pollen yield is from 10 up to 100 kg per ha (on average 40 kg) [Kołtowski 2006]. Lime trees that flower in the second half of June also lure insects with an abundance of honeydew produced by aphids.

The floral nectaries of *T. cordata* belong to open nectaries that are easily accessible to pollinating insects. Nevertheless, nectar production in lime is unreliable and strongly dependent on weather conditions during flowering. Good nectar production in these trees is promoted by warm and sunny weather as well as by abundant soil moisture. On the other hand, trees growing in the shade show poorer nectar production [Pogorzelec 2006, Lipiński 2010]. The few literature sources available show that the nectaries of lime are composed of numerous secretory trichomes which are located of the basal part of the sepals [Maksymiuk 1960, Shanmukha and Ramayya 1987].

In Poland *Tilia cordata* is one of the most commonly found tree species, valued not only for its high honey productivity, but also due to the medicinal properties of its flowers that contain many active substances (among others, flavonoids, essential oils, mucilaginous compounds, tannins, ascorbic acid, organic acids) [Ozarowski and Jaroniewski 1989]. Lime inflorescences (*Tiliae flos*) are used as a diaphoretic, anti-inflammatory and protective, sedative and diuretic agent in the form of infusions [Kohlmünzer 2000].

Due to sparse information on the structure of the floral nectaries of *T. cordata* and important role of its flowers in natural remedies the aim of the present, preliminary study was to investigate the structure of the sepals and secretory glands of this species using light and scanning electron microscopy.

**MATERIAL AND METHODS**

Flowers of *Tilia cordata* growing in a sunny place in the Botanical Garden of the Maria Curie-Skłodowska University in Lublin (51°14’53” N 22°34’13” E) were collected on 17 June 2010 at full bloom and in full nectar production. The floral nectaries were examined by light and scanning electron microscopy. Hand-cut sections were also prepared from fresh material containing the nectaries of lime.

**Scanning electron microscopy (SEM).** Sepals with the nectaries were fixed in a 2% solution of glutaraldehyde with 2.5% paraformaldehyde in 0.75 M phosphate buffer (pH 6.8) at temperature of 4°C for 12 h. Subsequently, the samples were dehydrated in an ethanol series and dried at the critical point in liquid of CO₂ in EMITECH 850 device. Using the sputter coater EMITECH K 550x, they were coated with gold. The preparations were examined under a TESCAN/VEGA LMU scanning electron microscope at an accelerating voltage of 30 kV.

**Light microscopy (LM).** The anatomical observations of the nectaries were based on semithin transverse sections, which were scanned under a Jenaval Contrast micro-
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scope. Sepal fragments with the nectaries were fixed in 2% paraformaldehyde and 2.5%
glutaraldehyde buffered at pH 7.4 in 0.1 M cacodylate buffer. Fixation was performed at
room temperature for two hours, followed by 12 h at 4°C. When fixed, the samples
were rinsed with 0.1 M cacodylate buffer at 4°C for 24 h and then treated with 1%
OsO₄. Subsequently, the samples were transferred to re-distilled water and stained with
a 0.5 M aqueous solution of uranyl acetate. After passage through increasing concentra-
tions of propylene oxide in ethanol and finally through pure propylene oxide, the sam-
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ples were embedded for 12 h in Spurr Low Viscosity resin at 70°C. The transverse sec-
tions were cut at 0.9 μm thick using a Reichert Ultracut-S ultramicrotome and a glass
knife, were stained with 1% methylene blue with 1% azure II in a 1% aqueous solution
of sodium tetraborate.

Hand-cut sections were prepared from fresh material containing the nectaries of
lime. The nectaries were tested for the presence of lipids with a saturated alcoholic
solution of Sudan III, for presence of phenolic substances with 10% FeCl₃, for presence
of starch with Lugol's iodine and for presence of polysaccharides (mucilage) with
0.02% ruthenium red.

Moreover, the length and width of 10 sepals from 10 different flowers as well as the
length of 10 secretory trichomes were measured in cross sections under a light micro-
scope, including the height and width of the trichome head and stalk, after one sepal
was sampled from 10 different flowers.

Statistical analyses. A standard deviation and the correlation coefficient were ana-
lyzed at the five percent level.

RESULTS

Five-sepalled ray flowers of small-leaved lime were characterized by a pleasant
scent and light yellow colour of the sepals and petals (fig. 1). The boat-shaped sepals
were 5.5 mm ±0.9 in length and 2.3 mm ±0.4 in width. Floral nectaries are located in
the basal part of the sepals (figs 2, 3). Nectar drops, accumulated in a depression of the
sepals, were well visible with a naked eye (fig. 2). Devoid of protoplasts, at places
dense, non-glandular hairs of different structure, length, and shape occurred in different
numbers on the fleshy, quite stiff sepals, likewise on the other parts of the flower
(figs 2, 3). Stellate type hairs (which branches all came out from one place), consisting
of 4–8 uniform cells (not shown), were observed on the lower surface (abaxial) of the
sepals, whereas two types of non-glandular hairs were found on the upper (adaxial)
surface of the sepals. There were shorter and twisted unicellular hairs on the sepal mar-
gins (figs 3, 4), whereas also unicellular, but much longer, whip-shaped non-glandular
hairs with a thinner cell wall occurred in the basal part of the sepals (figs 2, 3, 5, 6). The
whip-shaped hairs formed a dense silver-coloured tomentum and covered the nectary
located on the tongue-shaped projection of this part of the sepal (figs 2, 3, 5, 6, 7). This
projection and the remaining adaxial surface of the sepals were covered with an epider-
mis composed of polyhedral cells with a smooth cuticle without trichomes (figs 5, 8).

The trichomatos nectaries of lime were composed by numerous clavate, multicellu-
lar, glandular trichomes forming dense clusters on the projection of the basal part of the
The nectariferous trichomes of *T. cordata* with an average length of 88.3 μm ±7.1 consisted of 1–3 basal cells located in the epidermis, a 2–3-celled stalk with an average length of 35.3 μm ±6 and width of 15.3 μm ±2.7, and a multicellular head with an average height of 52.4 μm ±8.2 and width of 26.2 μm ±1.3, most frequently made up of 2–3 rows of secretory cells forming several layers (3–5) (figs 11–16). On the

Fig. 1. Flower of *Tilia cordata* at anthesis. S – sepal, P – petal

Figs 2, 3. The boat-shaped sepal of lime with two types of non-glandular trichomes: long and straight trichomes covering the nectary (stars) as well as shorter and twisted hairs borne on the sepal margin (arrowheads). Note a nectar drop flowing out (Nd)

Fig. 4. Short, twisted, unicellular non-glandular trichomes found on the sepal margin
basis of the value of the correlation coefficient (=1), it was found that the larger the length of the trichome was, the higher the height of its head was. The secretory cells were characterized by a dense cytoplasm with a large nucleus as well as they showed a varying degree of vacuolation but lower than the basal and stalk cells (figs 13, 15). The cuticle detached quite clearly from the cell wall of the secretory cells was observed in many places, especially at the tip of the trichome head (fig. 15). The occurrence of storage starch grains or the presence of lipids and phenolic compounds were not found in the trichome cells.

Underneath the secretory epidermis, there was a wide tier of nectariferous parenchyma made up of 7–9 layers of different shaped and tightly packed cells (figs 16–18).

Figs 5, 6. Fragments of the sepal of lime with a tongue-shaped projection (star) from which whip-shaped non-glandular hairs grow out (arrows) protecting the nectariferous trichomes (Nt) Note twisted non-glandular hairs borne on the sepal margin (arrowheads) (Fig. 6)

Fig. 7. The nectariferous trichomes with multicellular secretory heads

Fig. 8. Fragments of the adaxial epidermal surface of the sepal of *T. cordata* composed of tightly packed cells covered by a smooth cuticle
Figs 9, 10. Cross sections of the sepal of lime with visible clusters of secretory trichomes (stars), vascular bundles (arrows), and mucilage cavities (arrowheads).

Figs 11–14. Clavate-shaped nectariferous trichomes on the surface of the epidermis of the sepals of *Tilia cordata*. Fig. 12. Dorsal view; visible multicellular heads of the secretory trichomes. Figs. 13. Large vacuoles visible in the cells of the base and stalk of the trichomes. Large nuclei and a few and small vacuoles can be seen in the secretory cells of the trichome head. Nt – nectariferous trichomes, Hc – head cells, Sc – stalk cells, Bc – basal cells, E – epidermis.

Fig. 15. The secretory cells composing the heads of the nectariferous trichome of lime. At the tip of the trichome, a space can be seen between the cell wall and the detached cuticle (arrowhead).
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Fig. 16. Fragment of longitudinal section of the sepal with the trichomatous nectary. Visible nectariferous trichomes (Nt), the glandular parenchyma (Gp) with vascular bundles (arrows), and the subglandular tissue (Sgt) with mucilage cavities (Mc)

Figs 17, 18. Tightly packed cells of the glandular parenchyma (Gp) containing large nuclei and small vacuoles. E – epidermis, arrow – xylem vessel

Fig. 19. Cross-sectional fragment of the sepal of the *T. cordata* flower outside the nectary. Mc – mucilage cavity, E – adaxial epidermis, arrows – calcium oxalate crystals

The cells of this tissue were characterized by the presence of a large nucleus with a nucleolus and numerous, generally small vacuoles. In many cells of the secretory parenchyma, there were plastids containing small storage grains staining almost black with I-KI solution. The vascular bundles, which were located in the subglandular tissue, contained xylem and phloem and supplied the nectary, were adjacent to the glandular
parenchyma layer, while only xylem vessels were observed in the glandular parenchyma (figs 9, 10, 16, 18).

The subglandular parenchyma cells contain numerous chloroplasts and calcium oxalate crystals. Different-sized mucilage cavities staining purple-red with ruthenium red could also be seen in the subglandular tissue (figs 9, 10, 16, 19). The smaller cavities were of schizogenous origin, while those with a large diameter were formed lysiogenously (pieces of ruptured cell walls were visible in the cavities). In longitudinal sections of the sepal, it was noticed that single cavities did not run along the whole length of the sepal, but they ended in the subglandular tissue.

In the upper epidermal cells of the sepals, phenolic compounds were found to be present and they turned dark brown (almost black) when stained with FeCl₃. After the end of secretion, the sepals fell off together with the nectary trichomes.

**DISCUSSION**


The trichomatous nectaries in *Tilia cordata* are located at the base of the sepals where they form a densely packed cushion carpet covered by whip-shaped non-glandular hairs. The clavate, glandular trichomes consisted of a multicellular head, several-celled stalk, and 1–2-celled base. A similar location and structure of the nectariferous trichomes in other representatives of Malvaceae have also been observed by Findlay and Mercer [1971], Leitão [2005] and Goldberg [2009]. In *Tilia cordata*, at the tip of the active glandular trichomes there could be seen a space formed as a result of the detachment of the cuticle from the walls of the secretory cells due to the nectar accumulated. The absence of cracks in the cuticle suggests that it is probably permeable to nectar, and this phenomenon have also been described by Stpiczyńska et al. [2005] in *Hexisea imbricate* (Orchidaceae). A similar mode of nectar secretion has been observed in some representatives of Malvaceae; the nectar moved from the secretory parenchyma cells to the cells composing the base and the trichome stalk through the plasmodesmata to finally accumulate between the wall of the apical cells of the trichome head and the cuticle [Kronestedt et al. 1986, Sawidis et al. 1987a, Sawidis 1991]. Other authors report that in *Abutilon* and *Hibiscus* the nectar escaped beyond the detached cuticle through the microchannels in the cuticle and became accessible to pollinating insects [Findlay and Mercer 1971, Rocha and Machado 2009]. Other modes of nectar release
have also been observed in the family Malvaceae, notably through the decapitation of the apical cell [Kronestedt et al. 1986] or the bursting of the cuticle [Sawidis 1998].

In the subglandular tissue of the floral nectaries of *Tilia*, vascular bundles were found which contained xylem and phloem, while only xylem vessels were observed in the glandular parenchyma that supplied the nectariferous parenchyma cells with water and assimilates for the production of pre-nectar. According to other authors, the nectaries in many representatives of Malvaceae can be provided only with xylem, both with xylem and phloem [Gunning and Hughes 1976, Sawidis et al. 1987a, Leitão et al. 2002], or they can be provided with no conductive tissue elements [Rocha and Machado 2009].

The presence of storage starch grains in the cells of the secretory parenchyma of lime indicates that these starch reserves were the source of carbohydrates for the synthesis of pre-nectar components. Starch was formed some time before nectar secretion in the subglandular tissue, which is indicated by the presence of numerous chloroplasts in the cells of this tissue. During nectar secretion, starch is hydrolyzed and its products are used for pre-nectar production. Such relationships are reported by Pacini et al. [2003] and Nicolson et al. [2005].

The literature data show that in various species amyloplasts, chloroamyloplasts, amylchromoplasts as well as chloroplasts or proplastids can occur in the nectariferous tissue before secretion [Nicolson et al. 2007]. The results presented in this paper are initial results and they do not allow one to determine clearly what type of plastids accumulated starch in the secretory parenchyma cells of lime and what transformations they underwent after the end of secretion. Further transmission electron microscopy studies are necessary to analyse this differentiation and for the better understanding of the processes of nectar secretion.

Quite numerous deposits of phenolic compounds were observed in the upper epidermal cells of the sepals of *Tilia*. Large polyphenolic deposits in the cells of the nectar-producing trichomes of *Triumfetta* were also described by Leitão et al. [2002, 2005]. According to Ferreres et al. [1996], phenolic substances, which may also occur in the nectar, may repel pathogenic microorganisms and pests feeding on flowers. A property of polyphenols is also their autofluorescence capacity and this may in turn encourage bees, which can see UV radiation, to visit the floral parts “shining” in this way [Kevan and Baker, 1983]. In *Tilia* the location of phenolic compounds in the epidermal cells of the sepals, and not in the secretory trichomes, allows the autofluorescence capacity of these compounds to be better exposed, as these trichomes are shielded by dense nonglandular hairs which absorb UV radiation and do not transmit it.

The mucilage cavities, found quite numerously in the subglandular tissue in *Tilia*, can be an additional source of water required for nectar synthesis as well as necessary to increase in productivity and viability of the nectaries. Such relationships in the nectaries of *Hibiscus rosa-sinensis* are reported by Sawidis [1991, 1998]. According to Nobel et al. [1992], mucilage can bind 51 times its weight of water when hydrated in vivo. Other authors have also observed mucilaginous idioblasts, ducts, and cavities near the nectariferous tissue in various species belonging to the Malvaceae family [Sawidis 1998, Leitão et al. 2002, 2005, Craven et al. 2006, Rocha and Machado 2009].

Despite the fact that *Tilia* flowers hang downwards and that their nectaries belong to open ones and their nectar is watery, these plants have adaptations preventing its loss.
Finally, they have developed two types of non-glandular trichomes that protect the nectary against evaporation of the secretion and prevent it from flowing outside the flower. The occurrence of similar types of non-glandular trichomes in other representatives of Malvaceae is reported by Leitão et al. [2005] and Celka et al. [2006], while Weryszko-Chmielewska and Konarska [2006] and Weryszko-Chmielewska et al. [2007] have described the presence of trichomes protecting the nectar against evaporation in flowers of plants from other families (Rosaceae, Ericaceae).

CONCLUSIONS

1. Nectar is released from the trichomatous nectaries of *Tilia cordata* through the cuticle that is not ruptured, thus it is probably permeable to nectar.

2. The floral nectaries of lime are provided with vascular bundles containing xylem and phloem, while the cells of the secretory tissue contain storage starch grains, and this suggests that carbohydrates do nectar synthesis occurs outside the nectariferous parenchyma, probably in the subglandular tissue.

3. The non-glandular trichomes found near the nectary protect the nectar against flowing out beyond the sepal and against adverse effects of weather factors.

4. The mucilage cavities as an additional source of water may contribute to an increase productivity and viability of the nectaries.

5. The phenolic substances present in the cells of the sepals can act as repellents to some visitors (pests and pathogenic microorganisms) and attract pollinating insects.

REFERENCES


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WSTĘPNE BADANIA NAD STRUKTURĄ DZIAŁEK KIELICHA I NEKTARNIKÓW TRICHOMOWYCH W KWIATACH Tilia cordata Mill.


Słowa kluczowe: Malvaceae, włoski wydzielnicze, mikromorfologia, anatomia, zbiorniki śluzowe, substancje fenolowe

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