UNIVERSITY OF PÉCS

Doctoral School of Biology and Sportbiology

Nectar chemistry and floral biology of some Solanaceae species

PhD theses

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1. Introduction

The plant family Solanaceae comprises approximately 85 genera and 2800 woody and herbaceous species with a broad geographical distribution in the tropical and temperate climate zones, originating from Central and South America. Several Solanaceous species are economically important, including numerous ornamentals, as well as melliferous plants. Thus, it is important to clarify the apicultural significance of floral nectar yielding species.

The majority of Solanaceae taxa are dichogamous, enhancing cross pollination and fertilisation (allogamy), since there is no overlap between anther dehiscence and stigma receptivity. From the two types of dichogamy, proterogyny is characteristic for Solanaceous species (Mione and Serazo 1999, Mione et al. 2001, Sousa-Pena 2001, Stace 2004), when the female reproductive parts become active before their male counterparts.

In most genera of Solanaceae, the basal part of the ovary is surrounded by a ring-shaped nectary (nectar disc) (Jos 1967, Huber 1980, Darók 1984, Armstrong 1986, Bernardello 1987, Gulyás et al. 1990, Galetto 1991, Cocucci and Galetto 1992, Mione and Serazo 1999, Bernardello et al. 2000, Rodriguez 2000, Hunziker 2001, Stace 2004, Bernardello 2007, Farkas et al. 2011). The nectaries of Solanaceous species native to Hungary were described in detail by Darók (1984).

Floral nectars are aqueous sugar solutions, containing mono- and disaccharides, as well as organic acids, phosphatases, glycosidases, mineral salts, aroma components and vitamins (Maurizio 1960, Karthasova 1965, Baker and Baker 1983). The sugar composition of nectar is dominated by sucrose, glucose and fructose (Percival 1961), but it may contain other carbohydrates such as arabinose, galactose, mannose, gentiobiose, lactose, maltose, melibiose, trehalose, melezitose, raffinose and stachyose (Baker and Baker 1983), but also oligosaccharides (Percival 1961) and dextrins (Rychlik and Federowska 1963).

Several studies have been conducted regarding the inter-relatedness of flower morphology, nectary and nectar traits and the mode of pollination. In *Lamium* species (Lamiaceae) positive correlation was found between the size of the nectary and the amount of nectar (Gulyás 1967). Also in Solanaceous species, strong correlation was reported between nectary size and nectar production (Darók 1984). The size of the nectary was found to correlate with the size of the flower in *Nicotiana tabacum* varieties (Gulyás et al. 1990) and in representatives of the Bignoniaceae family (Galetto 1995). There is a mutualistic relationship between plants and flower visitors (Harborne 2001). If a pollinator is fixed on certain nectar traits, it will facilitate pollinator-mediated selection, which in turn causes ethological isolation,

which may result in species divergence or species maintenance (Grant 1994). Due to the frequency of pollinator-plant associations, pollinators play a key role in speciation (Ollerton 1996, Waser et al. 1996, Waser 1998). Pollinators are known to continuously adapt to flower morphology (Cresswell and Galen 1991, Neal et al. 1998, Schemske and Bradshaw 1999, Ippolito 2000, Galen and Cuba 2001), and react to flower colour (Waser and Price 1981, Jones és Reithel 2001) and floral parts that secrete nectar (Schemske and Bradshaw 1999).

Since several kinds of pollination modes are characteristic within Solanaceae, species of this family can serve as suitable model plants for analysing plant-pollinator relationships. Pollinators are attracted by prirmary attractans, mainly nectar and pollen. In the course of my investigations I have focused on nectar traits such as nectar volume, nectar sugar concentration and composition, and dynamics of nectar secretion. Secondary attractants include the colour and size (length) of the flower. We examined the latter character, in relation with the size of the nectary.

Several members of the Solanaceae family are used as raw materials in pharmaceutical industry, due to their alkaloid content. At the same time, they also pose a threat when ingested. Therefore, it is essential to clarify if Solanaceous species that accumulate alkaloids in their vegetative parts, are able to secrete these active compounds in their nectar, as well. The apicultural use of these species can be largely influenced (limited) by the concentration of toxic compounds in the nectar.

2. Aims

Several melliferous taxa from the Solanaceae family were used as model plants to answer the following questions.

- How and to what extent do Solanaceous nectar glands differ in various genera, with different modes of pollination?
- What kind of relationship exists between the volume and sugar concentration of nectar?
- What is the connection between the volume of nectar and the size (area, surface, volume) of the nectary?
- How is nectar volume related to corolla structure (depth)?
- Is there a correlation between the size of the nectary and and the length of corolla?
- How are pollinator types related to nectar traits (volume, sugar concentration and composition), size of nectary and length of flower?

- What is the concentration of alkaloids (tropane alkaloids or nicotine) in the floral nectar of *Brugmansia*, *Datura*, *Lycium* and *Nicotiana* species, where vegetative parts accumulate substantial amounts of alkaloids? Does the nectar contain proteins, as well? How do the above compounds affect the behaviour of pollinators and thus the reproductive success of the plant?
- How can we characterize the nectar secretion dynamics of *Cestrum*, *Lycium* and *Nicotiana* taxa, whose flowers regularly produce abundant nectar (ca. 2-20 µL per flower)? How does nectar removal affect the volume and sugar concentration of nectar?

3. Materials and methods

Studied plant taxa, location and time of studies

Studies were conducted on 13 species from 10 genera of the Solanaceae family. Taxa were chosen with diverse origin, including species with tropical, subtropical and temperate origin. Another criterion for selection was that the species differed with regard to flower size and morphology, as well as mode of pollination, facilitating comparative evaluations. Finally, the genera *Atropa, Brugmansia, Cestrum*, *Datura*, *Hyoscyamus*, *Lycium*, *Nicandra*, *Nicotiana*, *Physalis* and *Withania* were included in my studies. Measurements were conducted in the Botanical Garden of the University of Pécs in the years 2004 to 2006, in the months May to September. Flowers for each study were chosen randomly.

Studies on flower morphology and anatomy

To measure the length of the flower and the area of the nectary, 15 flowers were sampled in each species. Flower length was measured without the peduncle. For microscopic studies, flower samples were dehydrated in ascending acetone series, and embedded in paraplast. Medial longitudinal sections were cut in 8-10 µm thickness with a rotation microtome (Anglia Scientific). Sections were stained with toluidine blue and mounted in Canada balsam. Histological studies were conducted with a Nikon H600L Eclipse 80i type microscope, digital photos were taken with SPOT 4.0.4 software, and quantitative characers were measured with UTHSCSA Image Tool 3.0 program.

Determination of nectar volume and nectar sugar concentration

Floral nectar was extracted from the flowers and its volume was measured with glass capillary tubes bearing microliter marks (CM Scientific Ltd., Silsden, United Kingdom). For each species we sampled 70-80 flowers of 20-60 individuals (depending on the species) between May 2005 and June 2007. The sugar concentration of nectar was determined (as sucrose equivalent) with an ATAGO N-50E hand refractometer.

Determination of nectar sugar composition

Nectar samples were dissolved in 70 % (v/v) ethanol, up to a final volume of 200 µl. Nectar sugars were separated by high-performance thin layer chromatography (HPTLC), on plates Merck HPTLC™. Plates were developed twice in ethyl acetate : ethanol : 60% acetic acid, coldly saturated aqueous solution of boric acid (5:2:1:1). Glucose, fructose and sucrose (1 mg/mL) were used as standards. Spots were visualised with a thymol sulphuric acid reagent. Quantitative evaluation was done with densitometry (Camag Scanner II V3.15, CATS 3.14 software).

Quantitative and qualitative analysis of nectar proteins

The quantity of nectar proteins was measured with the method of Bradford (1976), using bovine serum albumin (BSA) as calibration standard. Nectar proteins were separated with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), following the method of Laemmli (1970). For each species, 15 µl of raw nectar was loaded in one pocket, using 15% (w/v) separating gel in mini-gel system (Bio-Rad™). Protein molecular weight markers (Fermentas™) were used as standards. The proteins were made visible by using PageBlue™ Protein Staining Solution-nal (Fermentas™).

HPLC analysis of nectar alkaloids

Nectar alkaloids were detected with high-performance liquid chromatography (HPLC). Nectar samples were diluted twofold, then filtered with a syringe filter (Millex-HN, 0.45 µm, Nylon, 33 mm, non-sterile, Merck Millipore, Darmstadt, Germany). HPLC analysis was performed using a Shimadzu liquid chromatograph [two pumps (LC-10ADVP), degasser (DGU-14A), manual injector with a 20 µL loop, diode array detector (SPD-10AVP) and a computer data acquisition station].

Study of nectar secretion dynamics

Nectar secretion dynamics was studied on 15-20 flowers per species in the field. Prior to the measurements and also between sampling occasions, flowers were isolated with a tulle net to exclude visiting insects. Nectar was not removed from the flowers preceding the measurements. On each day of the study, nectar volumes were measured hourly between 8 a.m and 6 p.m. with glass capillary tubes bearing microliter marks (CM Scientific Ltd., Silsden, UK), and sugar concentration (refraction) of the nectar was determined with a hand refractometer (Atago N-50E). In addition, the number of dehisced anthers was counted and receptivity of the stigma was recorded in each hour. Simultaneously, we measured air temperatures and relative humidity with a Testo 610 type instrument.

Statistical analysis

In order to provide a more comprehensive analysis, we completed our own data with literature data on nectar, nectary and floral traits. We decided to use secondary data as well, because the other studies were conducted in the original habitats of tropical and subtropical taxa (mostly in South and Central America). Comparing our data from the temperate region with data from the above studies allowed us to estimate the significance of climatic factors on nectar production and nectar traits.

In most cases data were analysed with Microsoft Office™ Excel. In addition to calculating the mean, standard deviation (SD) and standard error (SE), the analysis of nectar production studies included correlation coefficients and percentile values, too. The relationships of floral, nectar and nectary traits with each other and with pollinators were analysed with R statistical software (R Core Team 2013). In box plot analyses and correlation studies, pollinating vectors were treated in three groups. In case of nectar volume and nectary size 10-base logarithmic scale was used in order to normalize the data. Data from our measurements and from literature were compared by ANOVA between pollinator groups.

4. Results and discussion

Topography and anatomy of the floral nectary

In family Solanaceae and also in the studied species the ring shaped nectary is located at the basal part of the ovary. The gland is mostly automorphic, and depending on the genus, more or less protruding above the ovary wall. In this type, also the colour of the nectary is different from that of the ovary wall. In some genera, however, the epimorphic type is characteristic, when the nectary can be seen as the continuation of the ovary wall (Table 1). Typically, three regions can be distinguished within the nectary (epidermis, glandular tissue, parenchyma), but in some genera (e.g. *Cestrum, Hyoscyamus, Lycium, Physalis*) the nectar producing glandular cells are mixed with nectary parenchyma cells. Nectar is typically released through stomata, e.g. in *Brugmansia suaveolens* and *Datura stramonium*.

Table 1 Colour and morphological type of the floral nectary in Solanaceous species

Plant species	Nectary colour	Morphological type
Atropa bella-donna	orange	automorphic
Brugmansia suaveolens	brownish orange	automorphic
Cestrum × newellii	dark green	epimorphic
Cestrum parqui	dark green	epimorphic
Datura stramonium	light yellow	automorphic
Hyoscyamus niger	dark green	epimorphic
Lycium barbarum	yellow	epimorphic
Nicandra physaloides	yellow	automorphic
Nicotiana alata	brown	automorphic
Nicotiana rustica	bright red	automorphic
Nicotiana tabacum	brownish orange	automorphic
Physalis alkekengi	orange	epimorphic
Withania somnifera	light brown to orange	epimorphic

Flower length, nectary size, nectar traits and the mode of pollination

Statictical analyses revealed that nectar traits did not differ significantly in flowers visited by various pollinators. The flowers of butterfly and bird pollinated species were typically longer, their nectaries bigger, and they produced larger volumes of less concentrated nectar, with higher sucrose content, compared to bee and fly pollinated species, however, these differences were not significant in most cases. This could be due to the fact that standard deviations of nectar volume, sugar concentration, sucrose ratio and flower length data were large for species pollinated by the same type of pollinator, even within the same plant family.

There was no significant difference in the sugar composition of nectar between different pollinator groups, when analysing all the plant species that belonged to the same mode of

pollination. The size of the nectary can be considered as a conservative character, compared to nectar traits, which typically show large standard deviations even within a species.

Relationships between nectary and nectar traits

By analysing several representatives of Solanaceae, we found that the sugar content of nectar decreased in parallel with increasing nectar volumes. This relationship was significant only for our own data (linear model: $p<0.01$; $R^2 = 0.567$). When taking into consideration literature data, as well, the relationship between nectar volume and sugar percentage was no longer valid (linear model: $p=0.109$). The size of the nectary and the amount of nectar was also related to each other. In a hierarchy model, the volume of nectar was in the strongest relationship with the area of the nectary from the four variables that can characterise the size of the nectary (area, surface, width and volume). Statistical analyses of our own data revealed that species with larger nectary area produced larger volumes of nectar (linear model: $p<0.01$; $R^2=0.506$). When taking into consideration SD values, the relationship was even stronger (glsme model: $R^2=0.520$). The same relationship was valid when literature data were also included in the statistical analyses, along our own data (linear model: $p<0.01$; $R^2=0.518$). Based on our own measurements, species with larger surface nectaries secreted higher volumes of nectar (linear model: $p=0.0419$; $R^2=0.325$).

Relationship between flower length and traits of the nectar and nectary

The length of the flower was found to be related to the size of the nectary: in the species studied by us, the taxa with longer flowers had larger nectar glands (linear model: $p<0.0001$; $R^2=0.693$). Standard deviations were small for each variable, thus we found a correlation with similar strength when taking SD into account (glsme model: $R^2=0.685$). This relationship was valid also when literature data were included in the analysis (linear model: $p<0.001$; $R^2=0.689$).

Since there are few literature sources that discuss all floral, nectar and nectary traits investigated in our study, only the relationship of flower length and nectar sugar concentration could be analysed based on a dataset completed with literature data, in relation to pollinating agents. Statistical analysis revealed that in Solanaceae species the sugar concentration of nectar decreased with the increase of flower length, but only up to 2 cm length. Using only our own data we found a marginally significant relationship between nectar sugar concentration and flower length (linear model: $p=0.0542$; $R^2=0.297$). However, when including literature data in the analysis, this relationship was no longer significant (linear model: $p=0.165$).

Protein content of nectar samples

The nectar of each taxon studied contained proteins (Table 2). From Solanaceae species we were the first to detect proteins in the floral nectar of *B. suaveolens*, *C.* × *newellii*, *L. barbarum* and *N. rustica*. Earlier data on the protein content of nectar were reported for the species *C. purpureum*, *D. aurea*, *N. attenuata* and *N. tabacum* (Bezzi et al. 2010, Zha et al. 2012), in similar range as the concentrations measured by us. Protein content varied widely not only in different genera, but also within the same genus (*Nicotiana*). In accordance with protein concentrations measured by us, the strongest protein bands on the SDS-PAGE gel photo could be observed in *N. rustica*, which contained an order of magnitude higher concentrations of proteins compared to *N. tabacum*. Nectar proteins are important elements in pollinators' diet, while some amino acids (e.g. proline) in the proteins are essential for the flight of insects.

Table 2 Protein content of floral nectar samples

Plant species	*Brugmansia suaveolens*	*Cestrum* × *newellii*	*Lycium barbarum*	*Nicotiana alata*	*Nicotiana rustica*	*Nicotiana tabacum*
Concentration (µg/mL)	88	44	131	84.5	265.5	21

Alkaloid content of nectar samples

The HPLC analysis of floral nectar samples revealed that the nectar of each taxon investigated contained the alkaloid that is specific for the given genus (Table 3). We were the first to detect alkaloids in the nectar of *B. suaveolens*, *D. stramonium*, *L. barbarum*, *H. niger*, *N. alata* and *N. rustica*. Nicotine was identified in the nectar of all three *Nicotiana* species. Scopolamine was also detected in the other four genera studied, however, atropine concentrations were below the limit of detection. The concentrations of nectar alkaloids in *N. rustica* and *N. tabacum* can be toxic for honeybees on the basis of LD_{50} values (Detzel andWink 1993), whereas the nectar alkaloid content of the other taxa does not repel honeybees.

Table 3 Alkaloid content of floral nectar samples (mean ± SD) based on three parallel measurements

Plant species	**Alkaloid**	**Concentration (µg/mL±SD)**
Nicotiana alata	nicotine	0.79 ± 0.09
Nicotiana rustica		2.53 ± 0.14
Nicotiana tabacum		5.89 ± 0.40
Hyoscyamus niger	scopolamine	2.92 ± 0.13
Lycium barbarum		24.28 ± 4.89
Datura stramonium		99.01 ± 3.20
Brugmansia suaveolens		149.80 ± 6.01

Nectar secretion dynamics and its pollination biological significance

In *L. barbarum* the aging of the flowers, as well as increasing air temperatures and decreasing relative humidities resulted in decreasing nectar production. The highest values of nectar sugar concentrations were measured in the hottest hours. In *H. niger* a similar pattern was observed as in the previous species, except that nectar sugar concentration did not show any relationship with temperature and humidity values.

The nectar secretion dynamics of *C.* × *newellii* differed on the two days of the study. One of the main differences was that on the second day of the study there was less nectar in the flowers. This could be attributed to lower temperature values during the whole day, which resulted in the flowers aging more slowly, i.e. anther dehiscence took place slower.

From the species investigated, the nectar secretion rhythm of *N. rustica* was the most reliable, since flowers started to secrete nectar only at noon and in early afternoon. This was most probably due to the fact that flowers opened and started anther dehiscence only at this time of the day. The study of *N. tabacum* revealed that flowers produce nectar at night, as well, which is related to the nocturnal activity of the plant's natural pollinators (hawk moths and bats). Nocturnal nectar production studies led to similar results in another Solanaceae species, *Markea neurantha*, which is a bat-pollinated epiphytic shrub (Voss et al. 1980). Both in *N. tabacum* and its variety *purpurea*, nectar production reached its maximum on the second day of flower opening, but nectar volumes were slightly higher in var. *purpurea*. In the flowers of *N. tabacum* the amount of nectar decreased gradually during the night, in parallel with a decrease of nectar sugar concentrations.

In summary, in all six taxa studied, the volumes and sugar concentrations of nectar, as well as dehiscing anthers made the plants attractive for pollinators, and, together with stigma receptivity, enhanced effective pollination.

5. Summary

Our study of 13 Solanaceae taxa was organized around the following objectives: investigate the anatomy of the floral nectary; reveal the relationship between nectar volume and nectar sugar concentration and their connection with nectary size, length of corolla tube and type of pollinator; qualitative and quantitative analysis of main nectar constituents (sugars, alkaloids, proteins); reveal nectar secretion patterns in species with high nectar volumes (e.g. *Cestrum* × *newellii, Nicotiana tabacum*).

The ring-shaped floral nectary of the investigated Solanaceae species is located at the base of the ovary, the shape of the flower and the position of the nectary enhancing successful pollination. We provided the first description of the floral nectary in *Brugmansia suaveolens*, two *Cestrum* species, *Physalis alkekengi* and *Withania somnifera*. In the latter two species the elements of the glandular tissue and nectary parenchyma are mixed, creating a mosaic pattern.

We found positive correlation between nectary size and nectar volume, while nectar sugar composition did not correlate with any other nectar or nectary traits. The high level of variance in floral and nectar traits suggests that mean values are not always suitable for characterizing a plant species. Large variance in the above characters may have a strong effect on pollinators' preferences, since some pollinators prefer more reliable, less variable nectar sources.

We detected scopolamine for the first time in the nectar of *B. suaveolens, Datura stramonium*, *Hyoscyamus niger*, *Lycium barbarum* and nicotine in *N. alata* and *N. rustica*. Nectar alkaloid concentrations vary in a wide range even within the same genus: nicotine content of *N. alata* and *N. rustica* may be attractive or even addictive for bees, whereas that of *N. tabacum* can be aversive. Proteins were detected for the first time in the nectar of *B. suaveolens*, *C.* × *newellii*, *L. barbarum* and *N. rustica*. Proteins that are present in the nectar of each studied species may enhance pollination efficiency due to their nutritive property.

Our study of nectar secretion dynamics revealed that various taxa are affected differently by periodic nectar removal. We were the first to report the diurnal and nocturnal nectar secretion pattern of *N. tabacum*. Nectar volumes, sugar concentrations and anther dehiscence were found to simultaneously attract pollinators in all six taxa studied, thereby enhancing effective pollination at the time of stigma receptivity.

6. References

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7. List of scientific publications

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2. **Kerchner A**, Darók J, Bacskay I, Felinger A, Jakab G, Farkas Á (2015) Protein and alkaloid patterns of the floral nectar in some solanaceous species. ACTA BIOLOGICA HUNGARICA 66:(3) pp. 304-315. [Q3, IF:0.563]
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