

## New insights into the anatomy of an endemic *Hladnikia pastinacifolia* Rchb.

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**Abstract** – We studied the anatomy of a rare narrow endemic species belonging to the Slovenian flora – *Hladnikia pastinacifolia* Rchb. *Hladnikia* is a monotypic genus with distinct taxonomic position within the Apiaceae family. The anatomical characteristics revealed by light and fluorescence microscopy provided new insights regarding the pollen, leaf and root characteristics of *H. pastinacifolia*, improving the understanding of its biology and ecology. Pollination, drought tolerance, life cycle and unattractiveness to herbivores explain the species' persistence in time. Autofluorescence localized bioactive substances within secretory ducts and oil ducts.

**Keywords:** mericarp anatomy, petiole anatomy, pollen, root chronology, Slovenian endemic, stomata

### Introduction

*Hladnikia pastinacifolia* Rchb. is one of the best known endemic species in Slovenian flora. *Hladnikia* is a monotypic genus, which has a distinct taxonomic position within the Apiaceae Lindl. (Umbelliferae Juss.) family (ŠAJNA et al. 2012). Furthermore, *H. pastinacifolia* is found only on the Trnovski gozd karst plateau in western Slovenia where it has a very narrow distribution area of 4 km<sup>2</sup>. Because of its rarity, it has been placed on the Red List and is among the species mentioned in Annexe II of the Habitat Directive (COUNCIL DIRECTIVE 1992). The entire distribution area is included in the Natura 2000 network as a Site of Community Interest (ČUŠIN 2004). Molecular analyses showed that *H. pastinacifolia* is a Pleistocene survivor *in situ* and has widened its distribution little since the end of glaciation (ŠAJNA et al. 2012). However, the species is not a habitat specialist and can be found

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growing in various habitats such as stony grasslands, rock crevices and screes. Among habitats, screes are priority habitats listed in Annexe I of the Habitat Directive (BERN CONVENTION 1979). Since *H. pastinacifolia* is not a habitat specialist (WRABER 1990) it could be expected elsewhere in the Dinaric mountains further to the southeast (NIKOLIĆ and TOPIĆ 2004).

The concise description of *H. pastinacifolia* is based on SUŠNIK (1964). *Hladnikia pastinacifolia* is monocarpic annual or biennial plant (HEGI 1975), also characterized as monocarpic perennial species (REICHENBACH 1830–1833). Plants are herbaceous, 15–30 cm high with long lignified root. Plants form only basal rosettes of numerous glossy and leathery leaves for several years before flowering. The flowering stem is erect. At first only one compound umbel is formed and later the stem branches and forms several lateral compound umbels. A single umbel consists of twenty rays. The involucre and involucre are multifoliate. Petals are white, heart-shaped, 1 mm long. Pistil has a prolonged style, which ends with a claviform stigma. Stylopodium is shorter than the style and rounded. The fruit is a typical schizocarp, which separates into two mericarps 4 mm long and 2 mm wide. Seed testa and fruit pericarp are fused. Most of the seed is occupied by endosperm, embryo is small and underdeveloped. Cross-sections through mericarps show protruding ribs and numerous smaller oil ducts.

Knowledge about the biology and ecology of rare and protected species is essential for their protection. Furthermore, anatomical characteristics, especially fruit anatomy, are very important for the taxonomy of Apiaceae. The doctoral dissertation of SUŠNIK (1964) was the leading work dealing with *H. pastinacifolia* morphology, anatomy, and taxonomy, relying on the means available at the time. According to his interest in taxonomic questions, the most important contributions of his thesis were the determination of the chromosome number ( $2n = 22$ ) and the comparison of *H. pastinacifolia* fruit extracts with *Falcaria vulgaris* Bernh. and representatives of the genera *Oenanthe* L. and *Athamanta* L. with paper chromatography. Additionally, he described the morphology and placement of floral elements and the anatomy of the mericarp (fertile mericarp *sensu* SUŠNIK (1964), since he hypothesized that in each fruit only one mericarp contains an embryo as a consequence of a mutation). Further on, he studied the morphology of leaf lamina and its changes during the plant's development. Plant parts that had no taxonomic value were addressed very briefly. SUŠNIK (1964) reported results about morphology and anatomy in a descriptive manner, and hardly any measurements were made. Later research concerning *H. pastinacifolia* was mainly focused on syntaxonomy (MARTINČIČ 1958, MARTINČIČ 1961, POLDINI 1978, KALIGARIČ 1997, KALIGARIČ and POLDINI 1997, DAKSKOBLER 1998, DAKSKOBLER 2006). Recently, research into the species' ecology was undertaken (ŠAJNA et al. 2009, ŠAJNA et al. 2011, ŠAJNA et al. 2012), as well as population genetics and phylogeny (ŠAJNA et al. 2012).

SUŠNIK (1964) did not perform anatomical studies on *H. pastinacifolia*, and accordingly we have endeavoured to complete the anatomical investigation with the use of contemporary techniques. We focus on characteristics, which are ecologically important from the point of view of species persistence in time. We have described new characteristics concerning leaf stomata and gathered new information about pollen characteristics and root anatomy.

## Material and methods

Sampling quantities and sampling locations were determined by a permit issued by the Republic of Slovenia's Institute for Nature Conservation, which allowed us to sample only

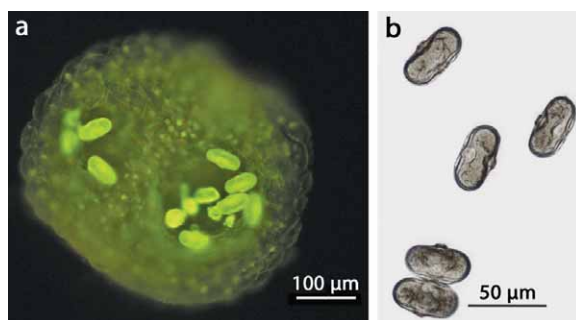
a few plants and their parts e.g. 30 leaves, 50 fruits. The proposed sampling location was a low-intensity pasture at Predmeja (Trnovski gozd, Slovenia). Mature fruits were sampled on 17<sup>th</sup> September 2004, leaves on 19<sup>th</sup> May 2005, and roots, as well as flowers on 28<sup>th</sup> June 2005. All voucher materials are preserved in FAA (formalin–acetic acid–alcohol), except dry fruits, and are deposited in the herbarium of the Faculty of Natural Sciences and Mathematics, University in Maribor (Slovenia). For the preparation of microscopic slides, we used fresh material or material fixed with FAA. Sections were performed by hand and observed in water. For the indication of root lignified tissues phloroglucinol dissolved in ethanol and concentrated HCl were used. During the histochemical reaction the cell walls, containing lignin, show a red coloration (DIETZ and ULLMANN 1998). We collected 3 roots from a flowering specimen. Part of the root 1 cm below the transition zone with the stem was sectioned. We studied the sections under a Nikon Eclipse 50i microscope. Additionally, we observed primary fluorescence of unfixed, unstained material with epi-illumination with blue light excitation. The source was a C-LHG1 Mercury Lamp HG 100 W. Autofluorescence in fruit sections (N = 10) was observed after 10 minutes of irradiation. Measurements under the microscope were performed with Eclipse Net software (Nikon, Waver, Belgium). To check whether in each fruit one mericarp fails to develop an embryo, we choose 30 fruits randomly from 6 main umbels.

We used hand-made epidermal peels to obtain micrographs for calculations of stomata density (SD; number of stomata per mm<sup>2</sup>), density of epidermal cells (ED; number of epidermal cells per mm<sup>2</sup>) and stomata index (SI% =  $SD/(SD + ED) \times 100$ ). We conducted one count for the abaxial and adaxial sides for 30 fully developed leaves, which had lobate laminae with 5 segments. We chose the lowest leaf segments to study. Leaf petiole was sectioned 5 mm before the first leaf segments.

We described pollen shape with the ratio of the length of the polar axis to the equatorial diameter (P/E ratio). By counting pollen grains of a single anther (N = 5), we quantified the number of pollen grains and calculated the pollen–ovule ratio (P/O ratio).

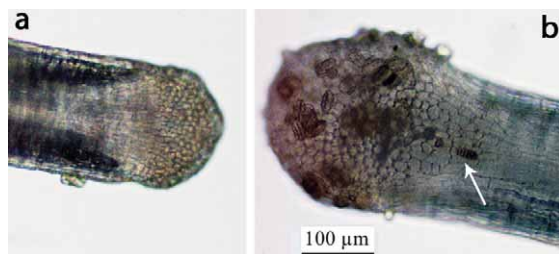
## Results

Flowers of *H. pastinacifolia* are protandric which means that the stamen ripens before the gynoecium at the time of anthesis. Pollen grains (Fig. 1) are trizonocolporate, oval,  $44.0 \pm 1.1 \mu\text{m}$  long and  $18.9 \pm 0.9 \mu\text{m}$  wide (N = 15). Pollen shape according to P/E ratio was



**Fig. 1.** Pollen grains of *H. pastinacifolia*. a – pollen grains inside the anther (autofluorescence of fixed material); b – trizonocolporate pollen grains.

$2.33 \pm 0.11$ . Inside the anthers, there were  $805 \pm 45$  pollen grains. Average P/O ratio for the whole flower with 5 stamens and 2 ovules was  $4025/2$ . At the time of anther maturity, both styles are positioned in parallel before the stigma becomes receptive (Fig. 2a). When the stigma does become receptive, it widens and takes on a claviform shape (Fig. 2b), while both styles edge away from each other.



**Fig. 2.** Micrographs of stigma shape during development. a – non-receptive stigma; b – receptive stigma with various pollen grains and fungal sclerotium (arrow).

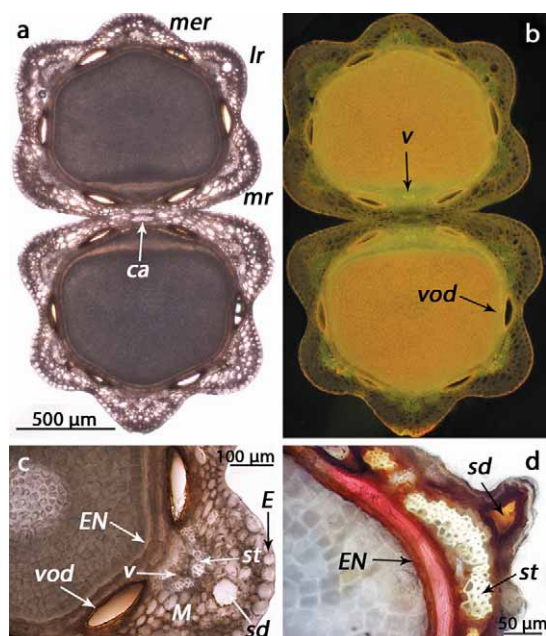
On the mature fruit the calyx teeth, style, and stylopodium remain present. At the time of maturity the schizocarp splits into two mericarps which remain attached to the carpophore (Fig. 3). The groups of sclerenchyma cells of the dual carpophore are positioned opposite each other in the ventral part of the commissure. The pericarp, measured between the ribs, is  $130 \mu\text{m}$  wide. The exocarp is formed of a single layer of isodiametric cells with a cutinized cell wall. The mesocarp is parenchymatous with 5 protruding ribs. Intrajugal oil ducts are round. Below them a sclerenchyma stereome with vascular tissue is found. Two vascular bundles are in each commissural half. Under the valeculae (depressions between ribs) six flattened oil ducts (vittae) are present. After the drying out of the mericarp at maturity, inside a rib the intrajugal oil ducts become tightly compressed and the secretory ducts shrink (Fig. 3d). In a dry mericarp, the vittae measure  $40\text{--}60 \mu\text{m}$  in diameter.

From longitudinal sections of mericarps we recognized an axial-linear embryo type (nomenclature after MARTIN 1946), which is narrow, flat and much longer than it is wide (picture not shown).

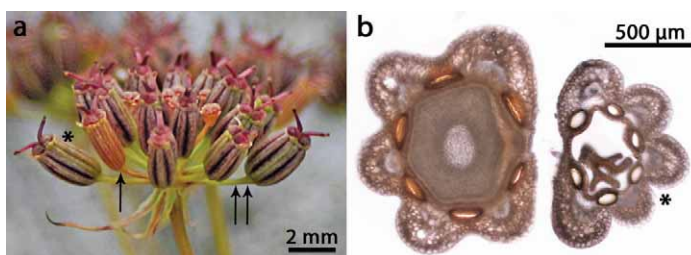
Fruits are homomeriarpic or heteromeriarpic if one mericarp does not develop properly (Fig. 4). Cross-sections of dry mature schizocarps from the main umbel showed that in approximately 60% of fruits both mericarps are fully developed and contain embryos. In 35% of fruits only one mericarp contains an embryo, while in 6% neither mericarp has an embryo. In a single umbellet all three combinations of fruits can be observed (Fig. 4).

Leaves of *H. pastinacifolia* are amphistomatal. Stomata are evenly distributed in upper and lower epidermis. Stomata are mostly of the anisocytic type, since one of the accompanying cells is smaller than the rest. Stomata of abaxial epidermis are  $34.6 \pm 5.5 \mu\text{m}$  long, while in the adaxial side they measure  $37.7 \pm 4.9 \mu\text{m}$  in length. Stomata density for the abaxial side is between 25 and 31 stomata per  $\text{mm}^2$ , ED was about  $113 \text{ mm}^{-2}$ , SI was on average 20%. Calculated values for the adaxial epidermis were:  $\text{SD} = 15 \text{ mm}^{-2}$ ,  $\text{ED} = 91 \text{ mm}^{-2}$ ,  $\text{SI} = 14\%$ .

Five to nine vascular bundles are found in leaf petiole (Fig. 5). Numerous secretory ducts are present in the petiole above and under each vessel, while a single secretory duct is pres-



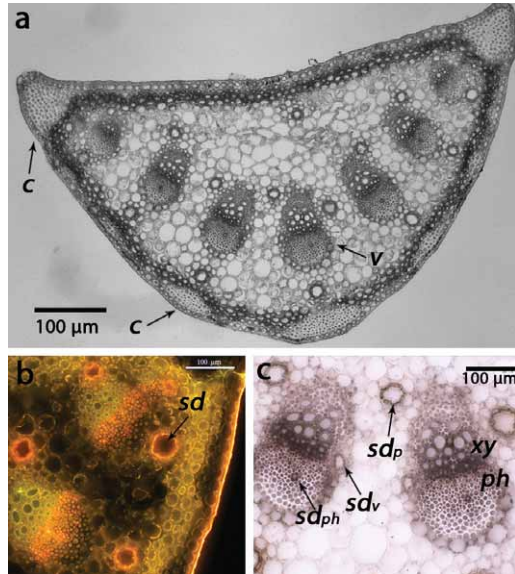
**Fig. 3.** Cross-section of *H. pastinacifolia* schizocarp. a – cross-section of an immature schizocarp; b – autofluorescence of immature schizocarp; c – detail of rib section; d – mature dry mericarp in detail (note the high accumulation of anthocyanins). Letters indicate marginal rib (*mr*), median rib (*mer*), lateral rib (*lr*), carpophore (*ca*), exocarp (*E*), mesocarp (*M*), endocarp (*EN*), secretory duct inside the rib (*sd*), valecular oil duct (*vod*), sclerenchyma tissue (*st*), vascular bundle (*v*).



**Fig. 4.** Variety of schizocarps in an umbellet of *H. pastinacifolia*. a – one mericarp does not contain an embryo (\*), both mericarps fail to develop an embryo (arrow), and both mericarps are fully developed containing embryos (two arrows); b – cross-section of a schizocarp with one fully developed mericarp and one without embryo (\*).

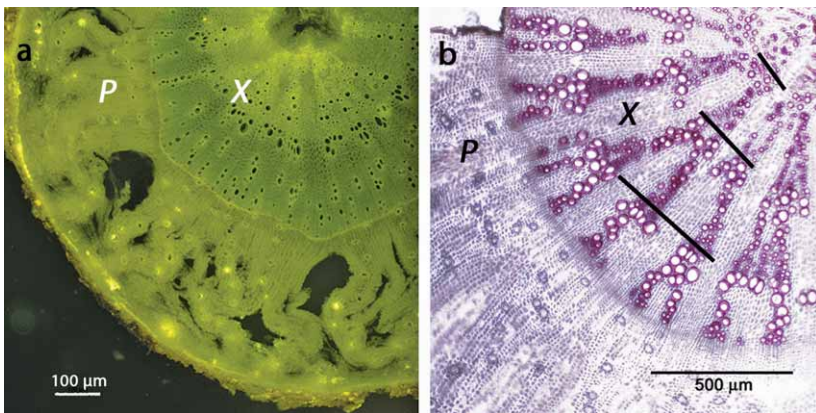
ent next to and a single one inside the phloem (Fig. 5c). Collenchyma is positioned sub-epidermally. The petiole parenchyma does not have any very big intercellular spaces.

Root cross-sections of *H. pastinacifolia* showed that secondary xylem and secondary phloem are well developed (Fig. 6). Secondary xylem exhibited characteristics that could be used for the age estimation of individuals, since we could distinguish delineated annual growth rings (Fig. 6b). We were able to recognize growth rings according to groups of large-



**Fig. 5.** Leaf petiole of *H. pastinacifolia*. a – cross-section showing 7 vascular bundles; b – autofluorescence of petiole showing yellow fluorescence of secretory ducts (*sd*); c – numerous secretory ducts are present in parenchyma (*sd<sub>p</sub>*); some accompany vascular bundles (*sd<sub>v</sub>*) and some are found within the phloem (*sd<sub>ph</sub>*). Letters indicate vascular bundles (*v*), collenchyma (*c*), xylem (*xy*), phloem (*ph*).

sized vessels formed in the beginning of each growing season. Therefore we could distinguish early wood and late wood. The rays of vascular elements were wide and often branched at the beginning of the growing season. Primary xylem in the center is followed by three annual growth rings (Fig. 6b). Therefore, we can determine that this specimen was 4 years



**Fig. 6.** Cross-section of *H. pastinacifolia* root. a – autofluorescence showing numerous bright yellow spots in the root cortex indicating essential oils inside secretory ducts; b – in the secondary xylem, according to phloroglucinol/HCl reaction with lignin, annual growth rings are visible (indicated by lines). Letters indicate secondary phloem (*P*), secondary xylem (*X*).

old at the time of harvesting. Older secondary xylem is lignified, while younger growth rings include much specialized xylem parenchyma. Secondary phloem does not form layers and numerous smaller secretory ducts are visible.

## Discussion

The new insight into *H. pastinacifolia* anatomy gave interesting new results which could help us to interpret the survival ability and fitness constraints of this rare taxon. The pollen-ovule ratio of *H. pastinacifolia* flower is 2013 on average. It has been stressed before that P/O ratio is inversely proportional to self-pollination within species (CRUDEN 1976). The umbels of *H. pastinacifolia* were frequently visited by insects because of nectar (excreted by stylopodium) and pollen. High P/O ratio leads to the conclusion that such flowers are visited by less effective pollinators and at the same time, they are less effective at self-pollination. Even though pollinators from the main umbel differed from those from the lateral umbels, flowering later in the season, the prevailing groups of pollinating insects were dipterans (Anthomyiidae and Syrphidae) and Coleoptera (own observation). Attraction of these groups of pollinators is explained by the low amount of sucrose in Apiaceae nectar (PETANIDOU 2005). Since many of the observed beetles were palinophagous, the high P/O ratio is beneficial for successful pollination of *H. pastinacifolia*.

SUŠNIK (1964) observed that some mericarps of *H. pastinacifolia* do not contain an embryo and concluded that this could be a fixed trait, which would add to species' rarity. However, our results show that mericarps without embryos are present in 35% of schizocarps. We did not find a reason for some mericarps not to develop fully, whether this is a consequence of ovule abortion or lack of fertilization.

Immature fruits of *H. pastinacifolia* accumulate anthocyanins in the endocarp (Figs. 3d, 4a). The mesocarp of one mericarp consists of 6 vallecular oil ducts and numerous secretory ducts associated with the synthesis and accumulation of biologically active substances and essential oils (ATIA et al. 2009). Autofluorescence microscopy reveals secretory tissues in Apiaceae fruits. ZOBEL and MARCH (1993) reported intense yellow autofluorescence of parts containing coumarins, furanocoumarins, and flavonoids. Oil ducts in *H. pastinacifolia* fruits show high yellow fluorescence, which probably determines the location of essential oils.

New findings include the presence of amphistomatal leaves in *H. pastinacifolia*. The stomata density in the abaxial epidermis is greater than in the adaxial epidermis. Amphistomatal leaves are a morpho-physiological and not a taxonomic plant trait. Such leaves are typical for plants from habitats with high irradiance since leaves do not grow horizontally according to light source, but at an angle (CARLQUIST and MILLER 1999). In all habitats of *H. pastinacifolia* high irradiance is characteristic for at least part of the day. Amphistomatal leaves are occasionally associated with dry habitats as well (PARKHURST 1978). The dark green and glossy leaves of *H. pastinacifolia* already visually indicate drought tolerance. Even though the Trnovski gozd plateau represents an orographic barrier resulting in very high precipitation, the measurements of soil humidity in the habitats of *H. pastinacifolia* in summer (data not shown) were very low, only 3–4%. In the beginning of July 2006, we observed smaller parts with dried out vegetation in stony pastures with *H. pastinacifolia*. According to our observation, microtopography was of key importance for the survival of plants from different species. This is also the case with *H. pastinacifolia*, since slightly ele-

vated parts had a lower density of vegetative rosettes and seedlings were absent. Therefore drought tolerance is an important trait that enables survival and persistence despite the generally prevailing humid conditions.

According to our results, *H. pastinacifolia* is a monocarpic perennial species. Time of flowering and subsequent dying of the plant occurs in plants older than 4 years. The distribution of vascular elements within *H. pastinacifolia* roots shows the annual formation of distinct growth rings, which could be used for the method described by DIETZ and ULLMANN (1997) to estimate a specimen's age. However, because root chronology is an invasive method and because in general, demographic characteristics like growth, probability of survival and flowering, as well as reproductive output of perennial plants are strongly size-dependent (WERNER 1975, METCALF et al. 2003), for the crude estimation of age the diameter of the root, measured with calipers, could be used in the field. The root of *H. pastinacifolia* is a storage organ which allocates resources to aboveground organs. Long and thick single roots, forming a rootstock, which grows deep, are characteristic for species which are better adapted to substrate coverage as well as to substrate erosion (MIYANISHI and JOHNSON 2007). Roots represent an important organ for persistence in the scree habitats of *H. pastinacifolia*, and also enable greater water uptake during dry conditions. The autofluorescence microscope observations showed the presence of numerous secretory ducts in the secondary phloem of *H. pastinacifolia* root, emitting bright yellow fluorescence. The considerable presence of autofluorescent substances, which could be attributed to essential oils and phenolic compounds, are most likely the reason we did not observe signs of herbivory on leaves and why fruits were rarely attacked by animals except Heteroptera.

Whenever we study extremely rare and protected species, we can face ethical and legal dilemmas because we have to interfere with populations and perform sampling, sometimes even with invasive methods (CERIANI et al. 2008). However, especially for such species, studies and knowledge are pivotal for their protection. In this paper we have shown that anatomical features can provide additional information for a better understanding of the species' biology and ecology. Our results about *H. pastinacifolia* contribute to a better understanding of species' pollination characteristics, drought tolerance, life cycle, and unattractiveness to herbivores.

### Acknowledgements

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