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<th>Journal:</th>
<th><em>Botany</em></th>
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<td>Manuscript ID</td>
<td>cjb-2017-0043.R2</td>
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<tr>
<td>Manuscript Type:</td>
<td>Article</td>
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<tr>
<td>Date Submitted by the Author:</td>
<td>06-Jun-2017</td>
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<tr>
<td>Complete List of Authors:</td>
<td>Weryszko-Chmielewska, Elżbieta; University of Life Sciences in Lublin, Department of Botany Chwil, Mirosława; University of Life Sciences in Lublin, Department of Botany</td>
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<td>Is the invited manuscript for consideration in a Special Issue?:</td>
<td>N/A</td>
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<td>Keyword:</td>
<td>trichomes, epidermis, furanocoumarins, histochemistry tests, <em>Heracleum sosnowskyi</em></td>
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Localisation of furanocoumarins in tissues and on the surface of shoots in Heracleum sosnowskyi Manden.

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Abstract: Heracleum sosnowskyi was introduced in Poland as a fodder plant. Currently, it is regarded as an invasive plant posing a health hazard to humans and animals and a threat to native flora. The aim of the study was to localise furanocoumarins in the stem and leaf tissues. The investigations were carried out using light, fluorescence, and scanning electron microscopy as well as histochemical assays. The epidermis of the analysed organs bears live, non-capitate hairs with variable length, which contain lipids, essential oils, polysaccharides, tannins, and furanocoumarins. The observations performed with scanning electron microscopy revealed the presence of a foamy substance and furanocoumarin crystals on the surface of the trichomes and other epidermal cells, as well as in the parenchyma cells. Characteristic furanocoumarin autofluorescence was present in the epidermis and on its surface as well as in the subepidermal parenchyma. Secondary fluorescence was emitted by furanocoumarins in different leaf petiole tissues: psoralen, bergapten, and xanthotoxin. We have detected for the first time the presence of furanocoumarins in different tissues of leaves in H. sosnowskyi. Furanocoumarins were present also abundantly on the epidermal surface of cells. This explains why the contact with the plant is dangerous to humans and results in development of photodermatoses.
**Key words**: epidermis, trichomes, furanocoumarins, histochemistry tests, *Heracleum sosnowskyi*.

**Résumé**: *Heracleum sosnowskyi* a été introduit en Pologne comme plante fourragère. Maintenant, il est considéré comme une plante invasive qui provoque des problèmes de santé chez les hommes et les animaux et qui constitue un danger pour la flore autochtone. L’objectif de cette étude a été de localiser des furocoumarines dans les tissus des tiges et des feuilles. Dans notre investigation, nous avons employé les microscopies photonique, en fluorescence, électronique à balayage, ainsi que des essais histochemiques. Dans l’épiderme des organes analysés sont présents des poils vivants de différentes longueurs qui contiennent des lipides, essences aromatiques, polysaccharides et tanins. Les observations en microscopie électronique à balayage faites sur la surface des poils et d’autres cellules de l’épiderme ainsi que sur les cellules du parenchyme ont démontré la présence de substances mousseuses et de cristaux de furocoumarines. Nous avons observé une autofluorescence caractéristique des furocoumarines dans l’épiderme et sur sa surface et dans le parenchyme hypodermique. La fluorescence secondaire a été émise par des furocoumarines, telles que le psoralène, le bergaptène et la xanthotoxine, dans de différents tissus des pétiloles. Pour la première fois nous avons détecté la présence de furocoumarines dans de différents tissus des feuilles de *H. sosnowskyi*. Les furocoumarines ont été présentes sur la surface des cellules de l’épiderme. Cela explique pourquoi le contact avec la plante est dangereux pour l’homme et génère la photodermatose.

**Mots-clés**: épiderme, poils, furocoumarines, essais histochemiques, *Heracleum sosnowskyi*.
Introduction

Depending on the taxonomic interpretation used, the genus *Heracleum* (Apiaceae) comprises 60 species growing mainly in the temperate zone of Eurasia (Szweykowscy 2003) or 120-125 species of *Heracleum sensu lato*, with 109 growing in Asia (Pimenov and Leonov 2004). In Poland, the hogweed *Heracleum sphondylium* is a native species with *H. sphondylium* subsp. *sphondylium* and *H. sphondylium* subsp. *sibiricum* distinguished as two subspecies. In the 70s of the 20th century, two species native to the Caucasus and Dagestan were introduced for cultivation: *H. mantegazzianum* Sommier and Levier as an ornamental plant and *H. sosnowskyi* Manden. as a fodder plant. Currently, these two introduced species are regarded as invasive plants in many European countries (Pysek and Pysek 1995; Nielsen et al. 2005; Moravcová et al. 2007; Tokarska-Guzik et al. 2012; Niinikoski and Korpelainen 2015). *Heracleum sosnowskyi* occurs in many localities in Estonia, the European part of Russia, Latvia, Lithuania, and Poland (Jahodová et al. 2007; Kabuce and Priede 2010; Baležentienė and Bartkevičius 2013).

Invasive *Heracleum* species growing in Poland produce high stems (2-5 m), differ in leaf morphology, produce many seeds, and adapt easily to various environmental conditions, in particular in areas where soil is disturbed. They colonize abandoned green areas, meadows, riverbanks, forest edges, and roadsides (Müllerová et al. 2013).

All *Heracleum* species contain furanocoumarins in their aboveground organs, which can be components of essential oils and exhibit photoallergic and photocarcinogenic properties. The most dangerous compounds are psoralen, bergapten, and xanthotoxin (Zobel and Brown 1990b, Dewick 1997; Jakubowicz et al. 2012). These furanocoumarins have been detected in many species from the families Rutaceae and Umbelliferae (Zobel and Brown 1990a). They are also present in some representatives of the families Moraceae - *Dorstenia* (Abegaz et al. 2004) and Fabaceae - *Psoralea* (Zobel et al. 1991).
In the presence of UV radiation, furanocoumarins cause photodermatoses in humans, which are manifested by redness associated with burns, swelling, blisters, and - consequently - persistent skin discolouration and scars (Wagner et al. 2002; Jakubowicz et al. 2012; Gulati and Guttman-Yassky 2014).

Environmental stress increased the production of furanocoumarins in *Heracleum lanatum*, *Apium graveolens*, *Ruta graveolens*, and *Bituminaria bituminosa* (Zobel and Głowniak 1994; Walker et al. 2012). In such circumstances, secretion of furanocoumarins on the surface of the analysed organs was enhanced as well. Furanocoumarins create a barrier on the organ surface protecting against the action of bacteria and fungi. Since these secondary metabolites have the ability to absorb UV rays, they may constitute a shield to protect the surface of plant organs against this type of radiation (Zobel and Głowniak 1994).

Many researchers report that furanocoumarins, like many other secondary metabolites, can serve as a defence against insect attack and control oviposition (Zobel and Brown 1995; Carroll and Berenbaum 2006; Zangerl et al. 2007). Enhanced biosynthesis of these compounds can be triggered by biotic factors, such as viruses, bacteria, fungi, and insects as well as abiotic elements, such as trace metals salts, antibiotics, sulphur dioxide, detergents, herbicides, and synthetic peptides (Głowniak and Kozyra 2001).

When washed away from the plant surface by rainfall, coumarins can also act as inhibitors of seed germination (Zobel and Brown 1995). The compounds inhibit cell division and plant growth (Hale et al. 2004).

Furanocoumarins are used in psoriasis therapy (Ceska et al. 1987; Conforti 2009; Martinez et al. 2010). Diwan and Malpathak (2007) describe that furanocoumarins exhibit a positive effect in the treatment of leukoderma, vitiligo, psoriasis, and multiple sclerosis and may be potent anti-HIV agents. Literature data indicate their role as potent antioxidants.
In addition to their diverse activities, furanocoumarins have been shown to have anticancer properties (Luo et al. 2011; Bronikowska et al. 2012).

Sosnowski’s hogweed, occurring in many regions of Poland, not only poses a threat to humans and animals but also, as an invasive plant, threatens the native flora and protected vegetation of National Parks (Zając and Zając 2001; Tokarska-Guzik 2005). The number of plant species was reported to decline 62-69% in dense *Heracleum* patches, in comparison with phytocoenoses without the presence of this plant (Sobisz 2007).

The control of invasive plants in large areas is associated with economic losses. In the buffer zone of the Góry Stołowe National Park in Poland, *H. sosnowskyi* occurs over an area of ca. 30 ha, which poses a serious economic problem (Tokarska-Guzik et al. 2012). Since the spread of *H. sosnowskyi* in Poland is a growing problem and cases of photodermatosis are still widespread (Wojtkowiak et al. 2008; Rzymski et al. 2015), we undertook investigations of the outer tissues of the stem and leaves in this species, which contain human health-threatening furanocoumarins. There are only few studies concerning the anatomical aspects of localisation of furanocoumarins. Zobel and Brown (1989) investigated the histological location of furanocoumarins in *Ruta graveolens* shoots: Zobel et al. (1991) in *Psoralea bituminosa* fruits, and Diwan and Malpathak (2010) in *R. graveolens* cell cultures.

This study is a continuation of our previous investigations of the furanocoumarin secreting structures in *H. sosnowskyi* (Weryszko-Chmielewska and Chwil 2014). The aim of the present study was to localise furanocoumarins in the leaf and stem tissues of this species with the use of light, fluorescence, and scanning electron microscopy as well as histochemical assays. Special attention was focused on structures present on the epidermal surface of the leaf, including trichomes and secretions that can be contained therein, as suggested by the literature on other furanocoumarin-containing species. The goals of this study were to determine: (i) whether furanocoumarins are released onto the surface of the aboveground
organisms via trichomes or other epidermal cells, and (ii) why even a short contact with the plant causes skin burns (photodermatoses).

Material and methods

Study material

Leaves of H. sosnowskyi Manden. were sampled from 5 plants in the Botanical Garden of Maria Curie-Skłodowska University in Lublin (51°15′49.76″N 22°30′49.59″E).

The leaves were collected from the 7th node of each plant. Cross sections for preliminary analyses were hand made from fresh leaf fragments (margin and central part of the lamina and petiole). The collected samples were fixed from SEM observations of the surface of the epidermis.

The epidermal surface and trichome structure were observed using stereoscopic (SM), bright-field light (LM), fluorescence (FM), and scanning electron (SEM) microscopy.

Microscopy

Stereoscopic microscopy

Preliminary observations of the epidermis and comparative analyses of the trichomes present on the lamina and petiole were carried out using an SMT 800T stereoscopic microscope. Photographic documentation was made with a Nikon Coolpix 4500 camera.

Fluorescence microscopy

Hand-made sections (3 – 4) x 5 plants of fresh leaf fragments were prepared. They were placed in a droplet of auramine O (Heslop-Harrison and Heslop-Harrison 1981). Slides were observed under a fluorescence microscope equipped with an FITC filter (EXP. 465-495, DM 505; BA 515-555). A UV-2A (EXP. 378/11; DM 416; BA 416 LP) filter was used for
localisation of flavonoids in the tissues after addition of aluminium trichloride or magnesium acetate (Charrière-Ladreix 1976) and furanocoumarins after treatment with 10% KOH in methanol (Harborne 1973). This filter was also used for analysis of autofluorescence of furanocoumarins. Microphotographs of the examined cells were taken with the Nikon Eclipse 90i fluorescence microscope coupled with a digital camera (Nikon DS-Mc) and a camera (Olympus C-7070). The furanocoumarin colour obtained was determined according to Maerz and Paul (1950).

**Bright-field light microscopy**

Trichomes, which differed in their structure and length, were observed on the cross sections of laminas and petioles collected from 5 leaves. Measurements of the trichome length were performed using the Nikon Eclipse 90i bright-field light microscope. Measurement details are presented in section 4 - Morphometric measurements.

**Scanning electron microscopy**

Fragments of leaves were fixed in 4% glutaraldehyde for 6 hours and in 0.1 M phosphate buffer, pH 7.0 at 4 °C for 24 hours. Next, the plant samples were dehydrated in a series of increasing acetone concentrations. After critical point drying in liquid CO₂, they were coater with a layer gold to a thickness of 20 nm using an Emitech K550X sputter coater. Micromorphological observations of the surface of the epidermal cells of the leaf were carried out using a Tescan VEGA II LMU scanning electron microscope.

**Histochemical assays**

The selected groups of biologically active compounds contained in the trichomes and cells of other tissues were determined based on staining reactions after using relevant
histochemical assays. Sudan III (Jensen 1962), Sudan IV (Pearse 1985), Sudan Red B (Brundrett et al. 1991), Neutral Red (Clark, 1981) were used to detect lipids and essential oils. Nile Blue was used to detect acid lipids and neutral lipids (Jensen 1962), Ruthenium Red for polysaccharides other than cellulose/pectins (Johansen, 1940), potassium dichromate (Gabe 1968) and vanillin/HCl-test (Gardner 1975) for tannins, aluminium trichloride and magnesium acetate for flavonoids (Charrière-Ladreix 1976), and 10% KOH in metanol (Harborne 1973) and Lugol’s solution (Broda 1971) for furanocoumarins. The presence of calcium oxalate crystals was determined using alizarin (McGee-RusSell 1958).

**Morphometric measurements**

We compared the length of the three types of trichomes on the surfaces of the petiole, midrib, and intercostal fields between the leaf veins (abaxial epidermis). The height of the trichome cell and the height of the epidermal cells surrounding the base of the trichome were measured separately. Sixteen measurements of each trait were performed in 3-4 sections from 5 leaves different plants. The measurements were carried out with the use of software for microscope image analysis Nikon NIS-Elements version 3.0 Advance Research. The mean of the measurements and the standard deviation were calculated using Microsoft Office Excel version 2013.

**Results**

**Micromorphology and distribution of trichomes**

The massive *H. sosnowskyi* shoots (Fig. 1A, B) had only non-capitate hairs classified in the literature as non-glandular trichomes. They were situated on the margins of the lamina (Figs 1C), on both leaf surfaces (Figs 1C, D, F), and they were more abundant on the abaxial
(Fig. 1F, G) than adaxial surface of the leaf (Fig. 1D). The trichomes were also present on leaf petioles (Fig. 2A-D) and stems (2E).

We distinguished 3 types of trichomes: Type I, II, and III (Table 1). Type I trichomes were composed of a long cell surrounded at the base by cells (0.4 – 9 mm) forming a high rosette (61-485 µm). The cell length in type II trichomes was 121 – 586 µm and epidermal cells surrounding the base were 20-101 µm high. Only a single cell (51-202 µm) was observed in type III trichomes and there was no rosette of epidermal cells at the base (Table 1, Fig. 2A, C). Trichomes were usually conical or cylindrical with a sharp apex (Fig. 2A-F). Trichomes with a rounded apex were noted infrequently. The largest petiole trichomes usually had a unicellular body and cushions composed of epidermal cells which were evidently elevated over the surface of other ca. 485 µm high epidermis cells surrounding the base of the trichome. The cells of the cushions contained anthocyanin (Fig. 2B, D). The cushions around the base of the trichomes located on the laminas (up to 60 µm high) were composed of 8-10 cells arranged in rosettes (Fig. 2F). The cuticle of some trichomes was verrucose (Fig. 2G).

**Secretions in tissues and on the leaf surface observed in SEM**

A foamy substance (FIG. 3A-C) or crystalline mass (Fig. 3D, E), most likely an accumulation of secretions, was visible on the surface of the trichomes or at their base. A foamy secretion (Fig. 4A, B) and crystal complexes (Fig. 4E, F) were also present on the surface of the epidermis of the leaf as well as stems (Fig. 3C). A foamy mass was also present among accompanied the crystal complexes (Fig. 4D, E). Similarly, the surface of the cross sections of the *H. sosnowskyi* petioles exhibited numerous crystals with a similar morphology (Fig. 4F, G). Identification of the crystals with Lugol’s solution confirmed the coumarin origin of the crystallised substance (purple-grey staining). Treatment with alizarin applied to
detect the presence of calcium oxalate crystals yielded a negative result, confirming the absence of this compound.

**Histochemical analyses**

Several classes of chemical compounds were identified inside the trichome cells. We detected the presence of lipids, essential oils, polysaccharides (pectins), tannins, and furanocoumarins. The presence of secondary metabolites, such as essential oils, flavonoids, tannins, and furanocoumarins, was also detected in the petiolar and laminar tissues.

Microscopic observations of the trichomes in the fresh plant material revealed the presence of viable transparent protoplasts with numerous granularities (Fig. 5A). Staining of the petiole cross sections with Sudan III, Sudan IV (Fig. 5B), and Sudan Red B revealed the presence of lipids (orange or reddish staining) in the epidermal cells and glandular epithelial cells (Fig. 5G). The use of Nile Blue confirmed the presence of acid lipids (blue staining) in some trichomes (Fig. 5C) and neutral lipids (pink staining) in others (Fig. 5D). The presence of lipids or essential oils in the trichomes was confirmed by the Neutral Red reaction (red staining) (Fig. 5E).

After staining with Ruthenium Red, which detects pectins, we observed a positive reaction in the trichomes (crimson staining). The presence of tannins both in the trichomes (Fig. 5F) and in the epidermal cells, subepidermal parenchyma, and around vascular bundles and secretory canals in the petioles (Fig. 5H) was detected with the use of potassium dichromate (brown staining). The presence of tannins in these tissues was also confirmed by their fluorescence emitted after application of the vanillin-HCl test (red staining) (Fig. 6D).

The yellow-greenish staining visible in these tissues after their treatment with aluminium trichloride (Fig. 5I) and magnesium acetate indicated the presence of flavonoids in the basal cells of the trichomes and in the subepidermal parenchyma cells of the petioles. The
presence of flavonoids in the parenchyma of the petiole was confirmed by yellow secondary fluorescence induced by magnesium acetate (Fig. 5J) and aluminium trichloride (Fig. 5K), which were used as fluorochromes for flavonoid detection.

Characteristic autofluorescence of furanocoumarins in the *Heracleum* tissues was observed under the fluorescence microscope. The typical blue psoralen fluorescence was visible in the epidermal cells and on their surface as well as on the surface of the trichomes (Fig. 6A, B). In turn, the yellow fluorescence of the subepidermal cells indicated the presence of bergapten (Fig. 6A).

The content of the basal zone of the trichomes and the adherent secretion exhibited green fluorescence after the auramine treatment (Fig. 6C). After the treatment of the fresh preparations with a 10% KOH solution in methanol, intense and diverse fluorescence of furanocoumarins was observed in the petiolar tissues of the leaf (Fig. 6 E). The epidermis and its surface as well as the epithelial cells around the secretory canals exhibited blue fluorescence (psoralen). The collenchyma and subepidermal parenchyma were characterised by orange fluorescence typical of xanthotoxin, whereas the parenchyma surrounding the secretory canals and vascular bundles exhibited yellow fluorescence characteristic for bergapten (Fig. 6E). See Table 2 for a summary of the histochemistry of the *H. sosnowskyi* tissues.

**Discussion**

**Types of trichomes**

In the present study, we have shown the presence of simple unicellular (non-capitate) trichomes composed of elongated, viable cells in the *H. sosnowskyi* epidermis of the leaves and stems. The longest trichomes were surrounded at the base by a multicellular rosette formed by epidermal cells. In accordance with the morphological typology proposed by Payne
The trichomes observed in *H. sosnowskyi* can be classified as “subulate”, (awl-shaped), and “attenuate” with a long, gradual taper. Cronquist (1981) and Judd et al. (2008) describe various sorts of hairs in Apiaceae (Umbelliferae). As suggested by Metcalfe and Chalk (1972), trichomes in representatives of the family Umbelliferae are nearly always non-glandular and can be divided into 5 types: (i) simple unicellular, (ii) unicellular, bladder-like, (iii) dendroid, (iv) stellate, (v) small glandular hairs with 2- and 4-celled heads. The trichomes in *H. sosnowskyi* examined in the present study belong to the first type specified by the authors; additionally, we distinguished three subtypes differing in the length and the presence or absence of a rosette at the trichome base. They are presented in Table 1. Epidermal cells in this type of trichome can have a rosette-like arrangement around the base of the hair; in such a case, they are referred to as “lateral” cells (Braune et al. 1979). In our previous study (Weryszko-Chmielewska and Chwil 2014), we observed some unicellular hairs on the epidermis of *H. sosnowskyi* leaves; they were surrounded by epidermal cells arranged in a rosette-like pattern and elevated above the surface of this tissue.

Hegi (1975) describes that the lower surface of *Heracleum* leaves bears either rough hairs or felt trichomes. As shown by our observations, the *H. sosnowskyi* trichomes can be regarded as the rough type. Similar findings concerning trichomes were reported by Arora et al. (1982), who examined leaves of other *Heracleum* species. They distinguished only unicellular thread-like trichomes of different lengths on the epidermis of *H. sibiricum* and *H. mantegazzianum*. The trichomes in these *Heracleum* species were also surrounded by 6-8 crown epidermal cells.

**Secretory activity of *Heracleum* trichomes**

The histochemical tests demonstrated that the trichomes present on the *H. sosnowskyi* shoots contained neutral and acid lipids, polysaccharides, flavonoids, tannins, and
furanocoumarins. We found differences in the content of these compounds for the individual
types of trichomes, which is shown in Table 2.

Under the light microscope, we observed irregularly shaped crystal complexes on the
surface of trichomes located on the leaves of the species. SEM revealed remnants of a
flocculent secretion on the outer trichome walls. Blue autofluorescence of the surface of the
trichomes, which is characteristic for some furanocoumarins (psoralen), was visible under the
fluorescence microscope. Based on the histochemical assays as well as the LM, FM, and SEM
observations, it can be assumed that the trichomes are viable and exhibit secretory properties.

Similarly, in their morphological, histochemical, and ultrastructural investigations of
non-glandular trichomes in 3 species from the family Lamiaceae and 4 species from the
family Verbenaceae, Tozin et al. (2016) demonstrated that the trichomes not only protected
plants against biotic and abiotic stresses but also, were capable as viable structures of
producing and secreting biologically active substances. As suggested by the authors, the
trichomes are involved in chemical interactions between plants and their environment. Other
studies have demonstrated that trichomes can accumulate toxic compounds (trace metal),
which are removed in this way from the plant (Choi et al. 2001; Lavid et al. 2001).

**Crystals on the surface of epidermis cells**

Phytochemical studies conducted by other authors showed that furanocoumarins not
only were present in plant organs but were also transported onto their surface. In species
examined previously, different percentages of the total furanocoumarin content on the surface,
of the epidermis of leaves were reported e.g. in *Ruta graveolens* 37 -56%, *Heracleum lanatum
82%, and *H. mantegazzianum* 1,9% (Zobel and Brown 1990b). Furanocoumarins were shown
to be able to crystallise (Zobel and Brown 1989; Gupta et al. 1993).
Scanning electron microscopy showed numerous crystal complexes and a foamy secretion on the surface of the epidermal cells of *H. sosnowskyi* laminas. The crystals were also visible under the light microscope. Cronquist (1981) reports that “crystals are rarely formed” in Apiaceae. Based on the negative result of the alizarin assay, we excluded the presence of calcium oxalate crystals. Numerous crystal complexes, similar to those observed on the surface of the epidermis, were also present in cross sections of *H. sosnowskyi* petioles within the parenchyma tissue, as shown by the SEM analyses.

Small furanocoumarin crystals on the surface of *Ruta graveolens* leaves were observed under a light microscope and SEM (Zobel and Brown 1988, 1989; Zobel and Głowniak 1994). Phytochemical analysis conducted by these authors revealed the presence of xanthotoxin, psoralen, and bergapten on the surface of rue leaves. Using SEM, Ceska et al. (1986) found long, needle-like furanocoumarin crystals, with angelicin as the main component, on the surface of *Pastinaca* (parsnip) roots (Apiaceae). They also detected other furanocoumarins such as 5-methoxypsoralen, 8-methoxypsoralen, psoralen, bergapten, and xanthotoxin in the mass of the examined crystals. Similarly, Zobel et al. (1991) observed complexes of needle-like furanocoumarin crystals on the surface of cotyledons in *Psoralea* seeds.

We assume that the numerous crystals on the surface of the *H. Sosnowskyi* leaves consist of furanocoumarins as well. We attempted to find the reason for such an abundant occurrence of the compound on the leaves of *H. Sosnowskyi*. Other authors found the greatest amount of furanocoumarin during the flowering period in two *Heracleum* species (Pira et al. 1989; Zobel and Brown 1990b).

A mycelium of a pathogenic fungus was visible on the leaves of the examined plants, as shown in the photographs. One of the functions of furanocoumarins in plants is protection of tissues against parasite invasion (Głowniak and Kozyra 2001). Krasawa et al. (1990) found
that the level of furanocoumarins in *Apium graveolens* plants infected by fungi was considerably higher than that in healthy plants. In the case of *Pastinaca* plants infected by fungi, Głowniak et al. (1999) demonstrated that the concentration of xanthotoxin, which has high antifungal activity, was higher on the surface of fruits than inside their tissues.

It seems that the flowering phase in the examined *H. sosnowskyi* plants may have caused the release of substantial amounts of crystalized furanocoumarins on the surface of the epidermis.

**Localisation of furanocoumarins in the plant**

Furanocoumarins produced in plant tissues have cytotoxic properties and, since they are dangerous for the plant, they are excreted (Podbielkowska et al. 1994; Zobel and Brown 1995). Their extrusion to the surface of the plant can serve as a barrier against herbivores, insect attack, bacteria, and fungi. When furanocoumarin excretion on the surface of the epidermis from deeper tissues is impossible, the compounds are transported to intercellular spaces (Zobel and Brown 1995). For example, we detected autofluorescent furanocoumarins in the intercellular spaces of the *H. sosnowskyi* leaf.

Zobel and Brown (1990a) demonstrated various concentrations of 3 furanocoumarins: psoralen, xanthotoxin, and bergapten on the surface of *Heracleum montegazzianum* and *H. lanatum* leaves. Xanthotoxin was present at the highest concentration in both species. It was followed by psoralen and bergapten in *H. montegazzianum* and by bergapten and psoralen in *H. lanatum*. Our histochemical assays have shown the presence, of these three furanocoumarins in *H. sosnowskyi*. We observed blue autofluorescence in the trichomes and on the surface of the leaf, which is typical of psoralen, as indicated by Zobel and Brown (1989). The subepidermal parenchyma in the leaf petioles exhibited orange fluorescence characteristic for xanthotoxin, whereas yellow fluorescence, which is typical of bergapten,
was detected in the parenchyma surrounding the secretory canals. We also observed green fluorescence on the surface of the epidermis and inside its cells. In their investigations of *Ruta graveolens* leaves, which exhibited green fluorescence, Zobel and Brown (1989) suggested that it was induced by the co-occurrence of the aforementioned furanocoumarins.

In plant material obtained from in vitro *Ruta graveolens* cultures, Diwan and Malpathak (2010) demonstrated that individual furanocoumarins were located in different tissues. Based on the characteristic autofluorescence, they found that the psoralen, bergapten, and xanthotoxin accumulation sites were spatially separated from each other. The authors found that psoralen emitting blue fluorescence was located in root initials and bergapten was identified in the epidermis of shoot initials based on its yellow autofluorescence.

In epidermal cells, furanocoumarins are deposited in the layer of epicuticular wax and on its surface (Zobel and Brown 1989, 1990a). The authors of those studies describe that vaporised furanocoumarins are detectable in the atmosphere surrounding rutaceous and umbelliferous plants. There are many known cases of photodermatoses triggered by direct skin contact with *H. sosnowskyi* leaves; however, photodermatoses were also found to develop without contact with plants but in their close proximity, where the concentration of coumarin volatile compounds in the air was high. These substances can easily penetrate the skin through the epidermis (Jakubowicz et al. 2012) and cause photodermatosis.

**Conclusions**

As shown in our study, there are only simple hairs different lengths on the leaves of *H. sosnowskyi*. The base of these trichomes is often surrounded by epidermal cells arranged in a rosette-like pattern. The trichomes are found in high density on the surface of veins on the abaxial surface of the leaves.

In the present study, we have shown for the first time secretion of furanocoumarins by *H. sosnowskyi* trichomes, whose surfaces were covered by a foamy substance or crystals.
Since one of the functions of trichomes is accumulation and disposal of redundant metabolites into the environment, furanocoumarins can also be removed via this route.

Using scanning electron microscopy, we have demonstrated the presence of furanocoumarin crystals not only on the epidermis of the leaf but also in the parenchyma of the petiole in *H. sosnowskyi*. The investigations conducted with the use of the fluorescence microscopy have shown that furanocoumarins (psoralen, xanthotoxin, bergapten) were contained in different *H. sosnowskyi* tissues. In this study, we have documented the presence of extremely numerous furanocoumarin crystals on the outer walls of the trichomes and epidermal cells of the leaf, which confirms the release of substantial amounts of these compounds on the surface of the plant and sheds light on the cause of the serious risk posed to humans who come in contact with the plant. The release of furanocoumarins from plant organs may suggest a great potential of *H. sosnowskyi* in protection against biotic and abiotic environmental threats, e.g. phytophages, bacteria, fungi, or ultraviolet radiation.

**References**


Figure captions:

Fig. 1. General habit of the plant and distribution of trichomes on the adaxial and abaxial leaf surfaces in *Heracleum sosnowskyi*. (A) Flowering plants produce large umbels composed of white flowers. (B) Smaller inflorescences grow in the axils of pinnate leaves. (C) Margins of laminas and veins are covered by trichomes. (D, E) Unicellular trichomes (arrows) are abundantly located on the margins of the lamina. (F, G) numerous trichomes on the margins of the lamina and veins, hyphae of a pathogenic fungus visible among the trichomes (double arrows). [Colour online.]

Fig. 2. Different types of trichomes on the epidermis of *H. sosnowskyi* leaves and stem. (A) Trichomes (arrow) of various lengths on the surface of the petiole. (B - D) Different types of trichomes on the surface of the leaf; multicellular base with anthocyanin (double arrows). (E) trichomes on the epidermis of the stem. (F) un-stained trichome from a fresh leaf with visible epidermal cells at the base (arrows) arranged in a rosette. (G) verrucose cuticle of the trichome surface (asterisk). [Colour online.]

Fig. 3. Trichomes with secretions on their surface and at their base. (A) Foamy secretion on the surface of the trichomes on the stem. (B, C) Foamy secretions on the surface of the trichomes on the leaves. (D) Crystals at the base of a trichome (asterisk). (E) trichome with green fluorescence and furanocoumarin crystals after treatment with auramine (asterisk). [Colour online.]

Fig. 4. Secretion (furanocoumarins) with a foamy and crystalline structure on the surface of the epidermis. (A, B) Foamy secretions on the cells (asterisks). (C) crystals (arrow) and foamy secretion (asterisk) on the surface of the epidermis of the stem. (D - F) Crystal aggregates (arrows) and foamy secretions (asterisks) on the surface of the epidermis. (G, H) Crystal complexes in petiole sections (arrows).

Fig. 5. Control leaf trichome, petiole trichomes, and cross sections of the petiole subjected to histochemical assays and fluorescence microscopy. (A) viable trichome with granularities visible in the cytoplasm. (B) Trichome stained red with Sudan IV (lipids). (C, D) Trichomes stained with Nile Blue: (C) blue indicating the presence of acid lipids (two asterisks), (D) pink indicating the presence of neutral lipids (asterisk). (E) Content of trichomes stained red with Neutral Red (lipids/essential oils). (F) Trichome stained brown with potassium dichromate (tannins). (G)
Petiolar tissues treated with Sudan Red; the content of lipids in epithelial cells around the secretory canals; epidermal cells around the secretory canals; epidermal cells were stained red revealing the presence of lipids/essential oils (arrows). (H) Petiolar tissues treated with potassium dichromate: epidermis and parenchyma cells stained brown (tannins). (I) Petiolar tissues stained greenish yellow after application of aluminium trichloride (flavonoids). (J) Yellow secondary fluorescence induced by magnesium acetate indicating the presence of flavonoids (double arrows) in the parenchyma cells of the petiole. (K) Subepidermal parenchyma cells exhibiting yellow secondary fluorescence after treatment with aluminium trichloride indicating the presence of flavonoids. [Colour online.]

Fig. 6. Fragments of petioles with primary and secondary fluorescence of tissues. (A, B) Epidermal cells and trichomes exhibiting blue autofluorescence typical of psoralen (arrow); (A) and yellow autofluorescence of the subepidermal parenchyma typical of bergapten (two arrows) (A); blue autofluorescence on the surface of the trichome indicating the presence of psoralen (B). (C) Parenchyma of the petiole cells exhibiting red secondary fluorescence of tannins (arrowheads); after the application of vanillin-HCl. (D) Petiolar tissues with intensified fluorescence after treatment with KOH: light blue indicating the presence of psoralen (white arrows), orange - xanthotoxin (two asterisks), yellow - bergapten (double arrows). [Colour online.]
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Table 1. Length of protective trichomes on the epidermis of *Heracleum sosnowskyi* Manden leaves.

<table>
<thead>
<tr>
<th>Analysed trait</th>
<th>Type of protective trichomes</th>
<th>Length of the trichome</th>
<th>Height of the epidermal cells forming a rosette at the base of the trichome</th>
<th>Height of the epidermal cells forming a rosette at the base of the trichome</th>
<th>Height of the epidermal cells forming a rosette at the base of the trichome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
<td>III</td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Epidermis of the petiole</td>
<td>I</td>
<td>2302.80-9009.20</td>
<td>3675.14±1526.47</td>
<td>202.00-484.80</td>
<td>306.16±99.22</td>
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<tr>
<td>Abaxial lamina epidermis</td>
<td>I</td>
<td>393.90-1030.20</td>
<td>651.45±187.04</td>
<td>60.60-161.60</td>
<td>109.21±29.32</td>
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<tr>
<td>Intercostal fields</td>
<td>I</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tbody>
</table>

Types of protective trichomes

I - long, bristly, sharply pointed trichomes located on high multicellular bases

II - medium-length sharply pointed trichomes surrounded by a several-celled base

III - short, blunt trichomes
Table 2. Results of histochemical tests of trichomes and epidermis and parenchyma cells in *H. sosnoswskyi* leaves.

<table>
<thead>
<tr>
<th>Staining procedure</th>
<th>Target compound</th>
<th>Colour observed</th>
<th>Intensity of colour staining in</th>
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<th></th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>trichomes</td>
<td>epidermal cells</td>
<td>parenchyma cells</td>
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<td></td>
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<td>(type II and III)</td>
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<td>+</td>
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<tr>
<td>Sudan III*</td>
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<tr>
<td>Sudan IV*</td>
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<td>red</td>
<td>++</td>
<td>+</td>
<td>-</td>
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<td>Nile Blue*</td>
<td>acid lipids</td>
<td>blue</td>
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<tr>
<td></td>
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<td>Neutral Red*</td>
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<tr>
<td>Vanillin + HCl**</td>
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<td>flavonoids</td>
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<td>+</td>
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<td>Aluminium trichloride**</td>
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<tr>
<td>Autofluorescence**</td>
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<td>+++</td>
<td>+</td>
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<tr>
<td>Auramine O**</td>
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<td>++</td>
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<tr>
<td></td>
<td></td>
<td>blue</td>
<td>+</td>
<td>++</td>
<td>+</td>
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<td>+++</td>
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<tr>
<td></td>
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<td>yellow</td>
<td>-</td>
<td>-</td>
<td>++</td>
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</tbody>
</table>

* - light microscopy, ** - fluorescence microscopy