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Flavonoid composition and localization in trichomes and leaves of *Degenia velebitica* (Brassicaceae)

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Abstract – *Degenia velebitica* (Degen) Hayek, the most important representative of the Croatian endemic flora, is a heliophyte and xerophyte highly specialized for survival in extreme living conditions characterised by intense solar radiation. In this work, the composition and *in situ* localization of flavonoids in the epidermal trichomes and leaf tissues of *D. velebitica* were investigated. Flavonoids were localized in the lumens of stellate trichomes, vacuoles and cell walls of epidermal cells, but only in peripheral parts of spongy and palisade parenchyma cells, on both the abaxial and adaxial sides of the leaf. Quercetin, kaempferol and isorhamnetin were the main flavonoid aglycones in extracts of trichomes and leaves. The results show the important physiological role of epidermal trichomes and flavonoids in protecting *D. velebitica* against insolation.

Keywords: fluorescence microscopy, isorhamnetin, kaempferol, quercetin, stellate trichomes, UV protection, xeromorphysm

Introduction

Degenia velebitica (Degen) Hayek is a monotypic plant genus from the Brassicaceae family and a stenoendemic in the Croatian flora, found in only a few locations along the Velebit and the Kapela mountains (Nikolić et al. 2015). It grows as a low, cushion-like plant whose vegetative parts are silver-gray (Fig. 1A). The branched, perennial rootstock bears numerous shoots, both fertile and sterile. The fertile shoots, up to 10 cm high, are unbranched, with densely packed leaves and a terminal inflorescence (Fig. 1B) of several relatively large flowers (up to 1 cm) intensely yellow in colour (Domac 1993). The sterile shoots are short, with a rosette of narrowly outlined leaves that are densely covered with trichomes, i.e. hairs (Fig. 1C). In July, the characteristic fruits are formed – ellipsoidal, spongy, swollen capsules, which are also silvery-gray due to the hair coverage (Fig. 1D). Upon ripening, the fruit opens and releases two flat seeds (Fig. 1E). With its characteristic and decorative leaves, flowers and fruits, *D. velebitica* is well known to amateur gardeners, professional growers, experts and botanists around the world, making it the main representative of the rich Croatian national flora and a symbol of its mountains.

Degenia velebitica was discovered by Hungarian botanist Arpad Degen during his botanical research on Velebit in July 1907; he clasified it in the genus *Alyssum* or *Vesicaria* (Degen 1909). The following year, Austrian botanist August Hayek concluded that it was a completely new genus and species in the European flora and named it *Degenia velebitica* (Hayek 1910) in honor of his colleague and its discoverer. Phylogenetic studies based on nuclear and plastid DNA sequence data nested *D. velebitica* in a clade consisting of five genera (*Alyssoides* Mill., *Clastopus* Bunge ex Boiss., *Degenia* Hayek, *Resetnikia* Španiel, Al-Shehbaz, D.A.German & Marhold, and *Physoptychis* Boiss.) within a monophyletic tribe Alysseae DC (Rešetnik et al. 2013). Because of its small population and distribution, estimated at 4.8 ha with 37,000 individuals (Liber et al. 2020), it has been a strictly protected species since 1964 and currently has the status of endangered species (Official Gazette 2013, Nikolić 2015-2025). Since it has a high percentage of seed germination (Naumovski 2005), classical cultivation has become the basis for *ex situ* conservation of this taxon as part of the Program for the Protection of Croatian Endemic Plant Taxa in the Botanical Garden of the Biology Department of the Faculty of Science in Zagreb (Stamenković et al. 2010).

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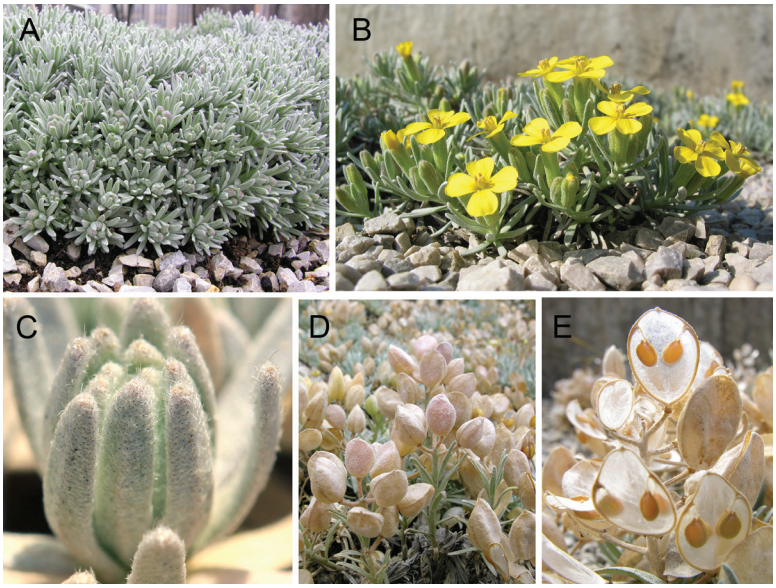

Fig 1. *Degenia velebitica*: A – plant habit, B – flowers, C – leaves densely covered with trichomes, D – ripe fruits, E – seeds.

Degenia velebitica grows in limestone screes and in rock crevices (Horvat 1930, 1931) exposed to intense insolation in summer and cold northeast winds in winter. Such extreme conditions define the ecological niche of this species, and account for its specific anatomical and morphological adaptations. Among the most important of these are the xeromorphic and heliomorphic structure of the leaves and stems including a dense coverage of stellate hairs, compact leaf mesophyll, numerous small stomata and short and thickened stem internodes, well-developed central conductive elements of the xylem and an extremely branched and long root (Stevanović and Vujnović 1990). Analysis of volatile compounds in *D. velebitica* leaves revealed eugenol, 2-methoxy-4-vinylphenol and benzyl alcohol as the main volatile O-aglycones, (9Z,12Z)-octa-9,12-dienic acid, hexadecanoic acid and phytol as the main free volatiles while the H_2O-soluble volatile fraction contained mostly glucosinolate degradation products and 3,4,5-trimethylpyrazole (Mastelić et al. 2010). However, more detailed research on the leaf structure and its flavonoid composition is lacking. Flavonoids are a diverse group of hydroxylated phenolic compounds with an aromatic ring structure performing a vast range of biological functions, including stress protection (Singh et al. 2021). The aim of this work was to investigate the anatomical characteristics of the *D. velebitica* leaf and the composition and localization of flavonoids in the leaf and epidermal trichomes as a posible protection against unfavorable environmental conditions.

Materials and methods

Plant material

Analyses were done on *D. velebitica* adult plants that have been grown from seeds in the Botanical Garden for several years. The daily solar radiation values, measured at the station for permanent monitoring Zagreb-1, which is close to the Botanical Garden, were from 5 W m^{-2} in the winter to the 45 W m^{-2} in the summer while temperatures were from -3 °C to 30 °C.

Leaf and hair structure

The basic anatomical features of the leaf structure were determined on medium-thick freehand transverse sections of fresh *D. velebitica* leaves incubated in a drop of 100 mM potassium phosphate buffer (pH 7.0). The sections were observed with an Olympus CX21 light microscope and the images were visualized and digitized using a DinoEye Eyepiece Camera (Dino-Lite, Netherlands) controlled by the Dinocapture 2.0 computer program. The shape, size, arrangement and surface composition of epidermal trichomes were determined using a scanning electron microscope (SEM) TS5136 (TeScan, Czech Republic) with an integrated energy dispersive spectrometer (Oxford, USA) and the associated computer program for visualization, digitization and data processing.

Localization of flavonoids by epifluorescence microscopy (EFM)

Flavonoid compounds in the leaves of *D. velebitica* were localized according to a modified method of Hutzler et al. (1998) and Tattini et al. (2000). Fresh leaves of the plants grown in the sun were transferred to the laboratory and immediately used for microscopy. Transverse and paradermal sections of the leaves were incubated in a drop of 100 mM potassium phosphate buffer (pH 7.0). The autofluorescence of individual structures was observed using a BX51 epifluorescence microscope (Olympus, Japan) with a WU-BP330-385 excitation filter (exc = 330 – 385 nm) and a BA-420 cutoff filter (em = 420 nm), and the micrographs were visualized and digitized using an Olympus DP70 digital camera controlled by the Olympus DP Controller 2002 pro-

gram. After observation of the autofluorescence, a drop of 0.5% (w/v) aqueous ammonia solution or 0.5% (w/v) 2-amino-ethyl diphenyl boronic acid solution ("Naturstoff" reagent, NR) in 100 mM potassium phosphate buffer was added. After incubation for 1 to 5 min, excess solution was removed by rinsing in the buffer. The secondary fluorescence of phenolic compounds after treatment with NR and fluorescence enhanced after alkalization with ammonia were observed at the same cross-section positions as autofluorescence.

Localization of flavonoids by confocal laser scanning microscopy (CLSM)

Flavonoids were also localized in medium-thick cross-sections of fresh *Degenia* leaves by a TCS-SP2-AOBS confocal microscope (Leica, Germany) at an excitation wavelength of an argon laser of 488 nm (exc = 488 nm) and 514 nm (exc = 514 nm) according to the method of Hutzler et al. (1998) and Agati et al. (2009). The leaf sections were treated in the same way as for fluorescence microscopy with the autofluorescence of the tissue observed before incubation and the secondary fluorescence after NR treatment. Detection of chlorophyll fluorescence was in the range 670 - 750 nm (exc = 514 nm), and flavonoid secondary fluorescence was in the range from 560 to 600 nm (exc = 488 nm) using the Leica Confocal Software 2.61 computer program.

Determination of flavonoids by high-performance liquid chromatography (HPLC)

Major flavonoid aglycones were analyzed in methanolic extracts of lyophilised leaves, detrichomized leves and trichomes incubated for 30 min at +80 °C and acidified with HCl until the final concentration of 5 M. Analytes were separated on a reverse-phase C18 Bischoff Zorbax ODS column (5 µm, 250 × 4.6 mm) with pre-column (5 × 4.6 mm) using a Perkin Elmer Series 200 system with a UV/VIS diode-array detector. The elution with mobile phase consisting of 20% (v/v) methanol with 0.5% (v/v) H_3PO_4 (A) and 100% methanol (B) was performed with a 28 min linear gradient from 100% to 25% A, 4 min linear gradient from 75% to 100% B and a 4 min isocratic with 100% B (Stamenković et al. 2015). The flow rate was 1 mL min^{-1}, and elution was monitored at 280 and 374 nm. Identification and quantification of flavonoid aglycones was made by comparing retention times in combination with UV spectral data using standard solutions of quercetin, isorhamnetin and kaempferol. The results were expressed as mg per gram of dry weight and shown as the mean value of at least three replicates ± standard error. For statistical analysis Fisher's least significant difference (LSD) post hoc test was used to compare flavonoid content in different tissues.

Results

General characteristics of leaf

The linear lanceolate leaves of *D. velebitica* are relatively thick, between 400 and 600 µm. The adaxial and abaxial leaf blades are densely covered with unicellular epidermal stellate trichomes – hairs (Fig. 2A, B). A single hair consists of a short stalk and numerous branches at the apex. The cell wall of a hair is markedly thickened, while in the interior of the stalk and branches there is a cavity without noticeable cell structures, but with visible droplet inclusions (Fig. 2C, D).

Beneath the layer of hair covering is a single layer of epidermal cells with moderately thickened cell walls (Fig. 2C). The cuticle is poorly developed and thin. Numerous stomata are located on both sides of the leaf (Fig. 2C). In the epidermis, enlarged cells with thickened walls are also visible – basal epidermal cells from which unicellular hairs develop (Fig. 2D). The mesophyll of the leaf (Fig. 2E) is differentiated into a very well-developed palisade parenchyma and a

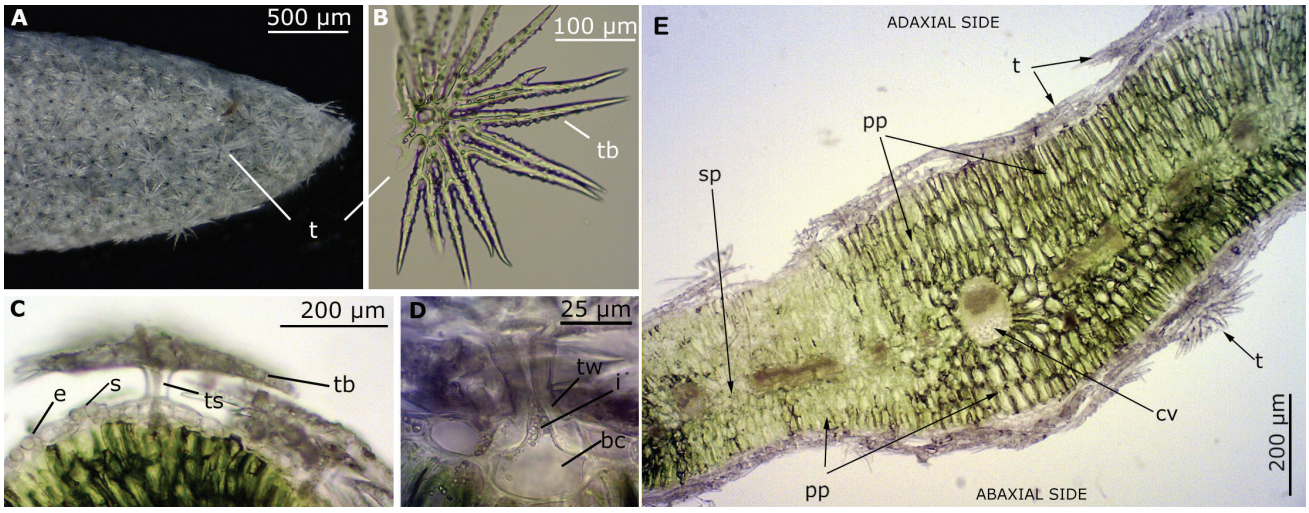

Fig. 2. Light micrographs of a *Degenia velebitica* leaf: leaf tip covered with stellate trichomes (A), isolated trichome (B), cross-section of the leaf margin (C) with enlarged detail of the hair shaft (D) and cross-section of a leaf (E). t – trichome, tb – trichome branch, ts – trichome stalk, tw – trichome cell wall, e – epidermis, bc – basal epidermal trichome cell, i – inclusions in the trichome lumen, s – stomata, pp – palisade parenchyma, sp – spongy parenchyma, cv – central vascular vessel.

less developed spongy parenchyma. The leaf is of the isobilateral type with three layers of elongated (cylindrical) palisade parenchyma cells on the adaxial side and two layers of palisade cells on the abaxial side. The spongy parenchyma is reduced and limited to the central part of the leaf, and is made up of several layers of small, round cells. The mesophyll cells, especially the palisade cells, are very compact, so the intercellular spaces are difficult to see. In the central part of the leaf there is a central conducting vein (Fig. 2E).

Morphology of epidermal trichomes by a scanning electron microscope (SEM)

The trichomes are densely arranged on the adaxial and abaxial sides of the lamina and cover the green parts of the leaf as a shield in at least two layers (Fig. 3A, B). The trichomes are of the stellate type, composed of a short stalk with a thickened slightly protruding center from which a dozen branches radiate, dichotomously divided into most often two (sometimes three) long tips. The tips of the branches are straight and very slightly bent to follow the leaf shape (Fig. 3B, C, D). The diameter of the upper, branched part of the hair is usually very large, up to 400 µm, so the hairs on the leaf surface are visible even to the naked eye. The adaxial surface of the trichome branches is tuberculate – covered with numerous small, elongated tubercles (Fig. 3E) up to 10 µm long, while the abaxial surface facing the epidermis is smooth (Fig. 3D, F).

The trichome stalks are short, 30 to 40 µm high and about 20 µm in diameter at the base. Micrographs of the stalk show a thickened, lignified cell wall and a cavity, which is filled with cell debris to a lesser (Fig. 3F) or greater (Fig. 3G) extent. Precipitated calcium carbonate crystals of non-biogenic origin were visible on some trichomes (Fig. 3F). Spectrometric analysis with a measurement point on the surface of the trichome showed high contents of oxygen (49.87%), carbon (38.08%) and calcium (12.04%).

Localization of flavonoids by epifluorescence microscopy (EFM)

In micrographs of the adaxial leaf section (Fig. 4A), the autofluorescence of cellular structures was clearly visible: light blue of the thick cell walls of the trichomes, bluish-green of the cuticle and outer walls of the epidermal cells with the rest of the cell being blue-violet. In the palisade cells, green fluorescence of cell walls, red fluorescence of chloroplasts and blue-violet fluorescence of vacuoles was observed.

After a 3-minute incubation with NR, secondary yellow fluorescence of flavonoids appeared having the most intensive signal within the cuticule (Fig. 4B) and guard cells of stomata (Fig. 4D). The epidermal cells showed intense yellow fluorescence of the walls and weaker fluorescence of vacuole content. In contrast, in the palisade parenchyma, yellow fluorescence was observed only in the peripheral parts of the cells corresponding to the cell wall, plasmalemma or cytosol while the blue-violet fluorescence of vacuoles and the red fluorescence of chlorophyll in the chloroplasts remained unchanged. In the trichomes the change in fluorescence occurred after 7-minute incubation with NR, with the central parts of the stalk and branches fluorescencing yellowish green, and the thick walls remaining blue (Fig. 4C).

On the transverse section of a leaf incubated for two minutes in ammonium hydroxide solution (Fig. 4E) an increase in green fluorescence of flavonoids was observed. Their localization partially corresponded to the sections

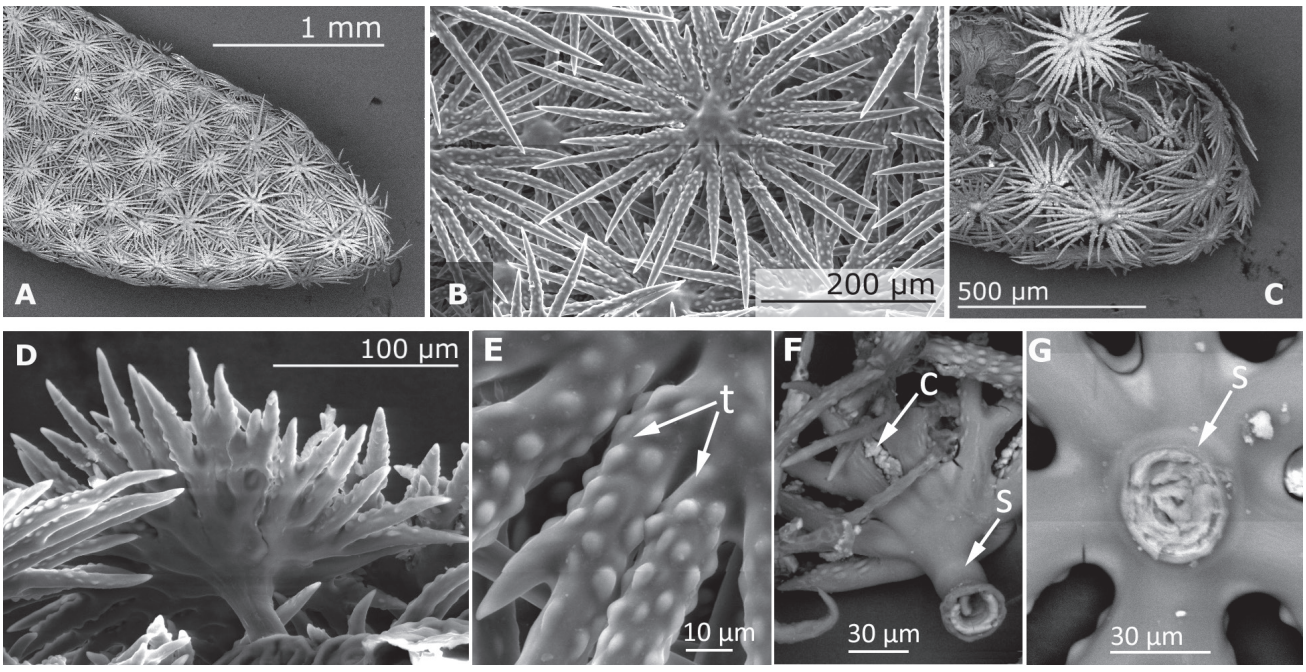

Fig. 3. Scanning electron micrograph of epidermal trichomes of *Degenia velebitica*: leaf tip covered with trichomes (A), trichomes from above (B) and from the side (C), trichome adhered to epidermis (D), tuberculate adaxial surface of trichome branch (E), trichome stalk from the abaxial side (F) and basal part of trichome stalk (G). t – tubercle or nodule, s – trichome stalk and c – calcium carbonate crystals.

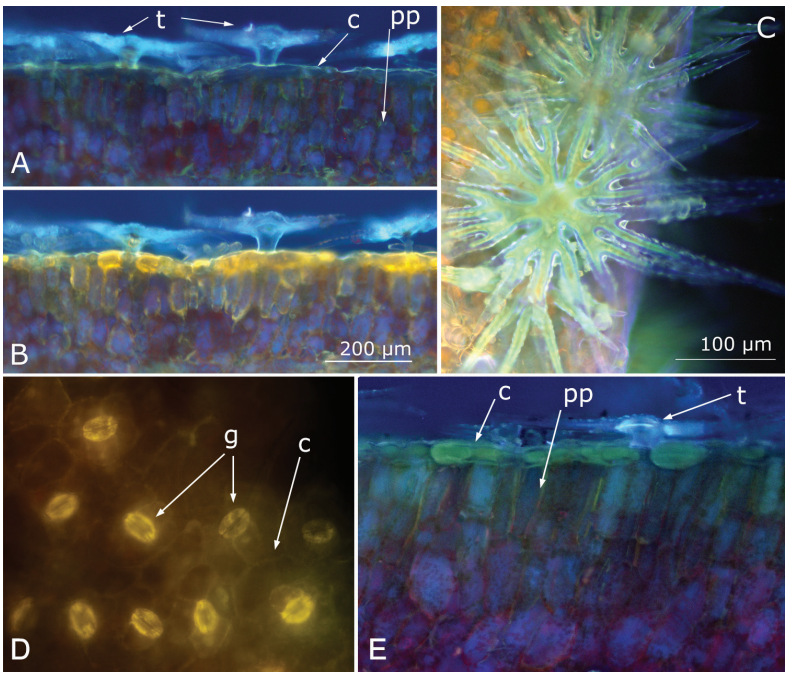

Fig. 4. Histochemical visualization of flavonoids in *Degenia velebitica* leaves by epifluorescence microscope: the autofluorescence of trichomes, epidermis and upper layers of palisade parenchyma in untreated leaf (A), secondary yellow fluorescence of the same tissue section (B), trichomes (C), and paradermal leaf section with guard cells (D) after incubation with "Naturstoff" reagent, and induced green fluorescence of epidermal and palisade cells after incubation with ammonium hydroxide solution (E). t – trichome, c – cuticle, e – epidermis, pp – palisade parenchyma, g – guard cell.

treated with NR – the epidermal cells fluoresced most intensely, but in addition to the fluorescence of the cell walls, intense fluorescence of the vacuolar contents of almost all cells was also visible. Cells of the upper layers of the palisade parenchyma in the area of the cell wall and cytosol also fluoresced, especially on the adaxial side of the leaf.

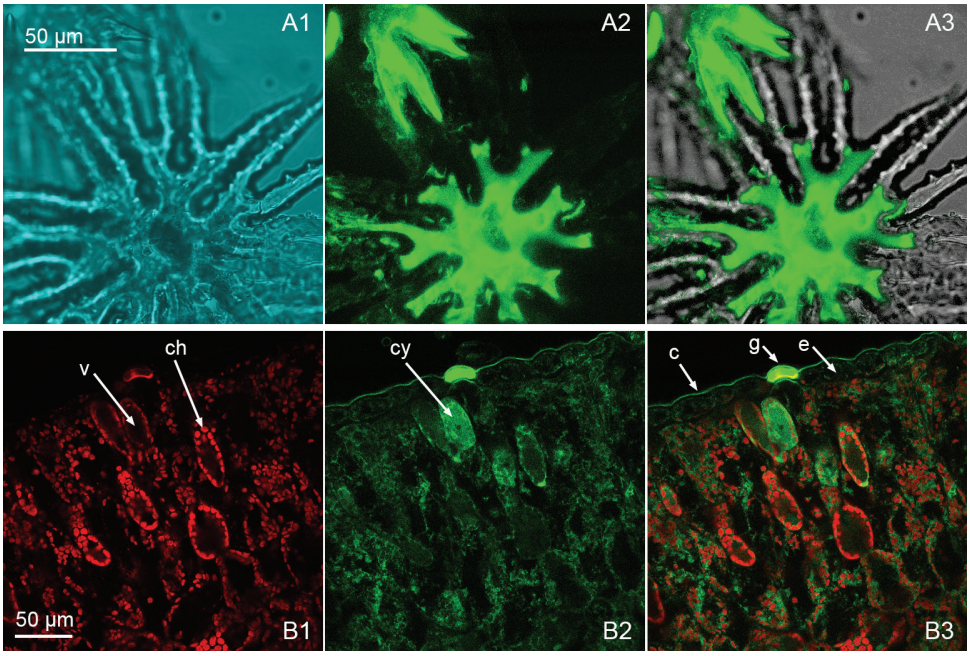

Fig. 5. Histochemical visualization of flavonoids in *Degenia velebitica* trichomes and leaf tissues by confocal laser scanning microscopy: the trichome in bright field (A1), the secondary fluorescence of flavonoids inside the trichome in pseudo-green color after the overlapping of seven separate micrographs that were obtained by scanning at different depths (A2), and an overlapping image of the previous two (A3); cross-section of a leaf first excited for autofluorescence of chlorophyll and visualized in pseudo-red color (B1), then excited at 488 nm after incubation with "Naturstoff" reagent, and monitoring the secondary fluorescence of flavonoids visualized in pseudo-green color (B2) and after overlapping the images of the two channels (B3). c – cuticule of the outer epidermal cell wall, ch – chloroplasts, cy – cytoplasm, e – epidermis, g – guard cells, v – vacuole.

Localization of flavonoids by confocal laser scanning microscopy (CLSM)

Secondary fluorescence of flavonoids was visible in the interior of the stalk and branches of epidermal trichomes that were scraped from the surface of fresh leaves and incubated with NR for 5-6 minutes. The most intense signal was visible in the area of the stalk and in the initial, wider parts of the canal of the branches (Fig. 5A2, A3).

The micrographs of leaf sections incubated with NR show cuticle (Fig. 5B2, B3) with an intense yellow fluorescence, in contrast to the epidermal cells, where the fluorescence was weaker. The most intense signal was observed in guard cells, where all parts of the cells fluoresced uniformly. The palisade cells had clearly visible chloroplasts (red fluorescence, Fig. 5B1, B3) located around a large, central vacuole while the secondary flavonoid fluorescence was visible in the peripheral area of the cells between the vacuole and the cell wall (Fig. 5B2, B3). No significant fluorescence was observed in the vacuoles.

Determination of flavonoids

Acidic methanolic extracts of *D. velebitica* leaves and trichomes were analysed by HPLC to detect flavonoid aglycons resulting from acid hydrolysis. In both extracts, one major peak at retention time 23.99 min and two minor peaks at retention times 26.81 and 27.4 min were detected (Fig. 6A).

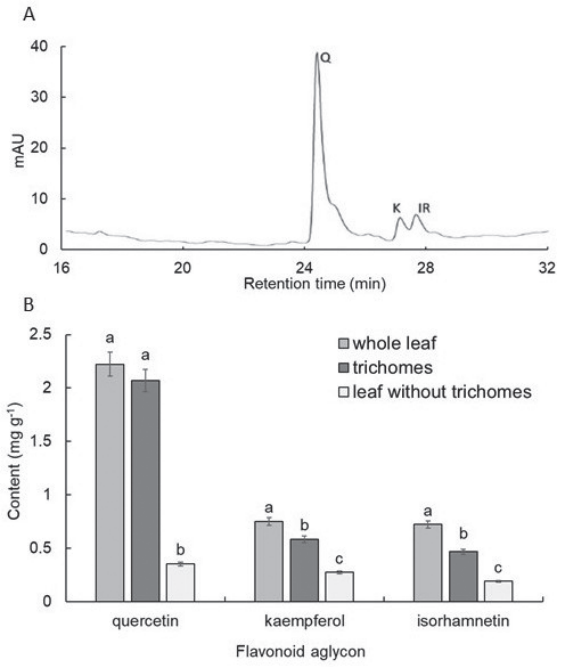

Fig. 6. *Degenia velebitica* flavonoid aglycones: chromatogram of acidic methanolic leaf extract showing peaks of quercetin - Q, kaempferol – K and isorhamnetin – IR (A) and content of flavonoids measured in whole leaf, trichomes or leaf with removed trichomes (B). The results are the mean value of three replicates ± standard error. Different letters indicate a significant difference between flavonoids in whole leaf, trichomes or leaf with removed trichomes according to LSD test ($P \leq 0.05$).

According to the retention times and UV spectra of pure standards the major peak was identified as quercetin while the two minor peaks were identified as kaempferol and isorhamnetin. Quantification showed that leaves and trichomes mostly contained quercetin, while kaempferol and isorhamnetin were less represented (Fig. 6B). Flavonoids were most abundant in trichomes. The concentration of quercetin in the trichome extract was even six times higher than that in the extract of leaves from which the hairs were removed. Also, the trichome extract contained two times more kaempferol and 2.5 times more isorhamnetin than the extract of detrichomized leaves.

Discussion

The leaves of *D. velebitica* are equifacial and amphistomatic, densely covered with epidermal trichomes (hairs) on both sides, which is visible even macroscopically. The stellate hairs are branched forming as many as two protective layers through which the epidermis is almost not visible. They are dead, filled with air and silvery-white because of the total reflection of light. Non-glandular trichomes are always composed of dead cells and this »hairy layer« prevents the sun falling directly on the epidermis, reducing its temperature which consequently reduces the level of transpiration (Dunkić et al. 2001). These anatomical features are typical of many xerophytes and heliophytes, i.e. they reflect the adaptation of plants to life in dry habitats with extreme insolation (Fahn and Cutler 1992). Trichomes are one of the first lines of defence against abiotic stresses such as UV exposure, water loss, temperature extremes and herbivore damage (Watts and Kariyat 2021). Similar morphological and anatomical features of stellate trichomes have been identified in leaves of the closest relatives within the tribe Alysseae, *Alyssoides utriculata* (Ančev and Goranova 2006) and *Resetnikia triquetra* (as *Fibigia t.*, Damjanović and Stevanović 1993). Brassicaceae trichomes are diverse in morphology, including malpighiaceous (or T-shaped) trichomes and stellate trichomes which could be radiate-stellate or peltate-stellate trichomes with dendritic branches as in the tribe Alysseae and are associated with arid habitats (Karabourniotis et al. 2020). Trichomes of *D. velebitica* have thickened, lignified walls and a spectrometric analysis in our study determined a high percentage of oxygen, carbon and calcium, which indicates the presence of calcium carbonate. It is known that silica, calcium carbonate and calcium phosphate play a prominent role in the biomineralization of trichomes, and the Brassicaceae family has trichomes mineralized with both calcium carbonate and calcium phosphate (Hopewell et al. 2021). Across different species of Brassicaceae from the tribe Alysseae to which *D. velebitica* belongs, the most common trichome was radiate-stellate with forked to dendritic branches, usually with a very rough surface and mineralized with calcium carbonate (Hopewell et al. 2021).

In cross-sections of *D. velebitica* leaves, the light blue autofluorescence (primary fluorescence) observed by EFM in

thickened trichome walls, cell walls of outer epidermal cells and cuticle originates from UV-excited polyphenolic compounds such as lignin (Sylvester et al. 1991). The intense yellow fluorescence observed by EFM in the central cavities of the hairs when the cross-section is excited with UV light after 7 min incubation in NR is evidence of the presence of flavonoids. Flavonoids play many roles in plant adaptation to changing living conditions, including the regulation of growth and development, active antioxidant activity and removal of reactive oxygen species (ROS) from the cell as well as attenuation of harmful UV radiation (Agati and Tattini 2010, Shen et al. 2022). Naturstoff reagent, i.e. 2-amino ethyl diphenylboronic acid, is a fluorescent dye that forms specific adducts with flavonoids, whose absorption-emission spectra are shifted towards longer wavelengths resulting in yellow to yellow-green secondary fluorescence (Hutzler et al. 1998). For the flavonoid localization in living plant tissues using NR, the most appropriate incubation sample time was from 1.5 min (Tattini et al. 2000) to a maximum of 5 min (Hutzler et al. 1998, Agati et al. 2009). The longer incubation time required for the appearance of secondary fluorescence in *D. velebitica* trichomes can be explained by the difficulty of NR penetration into the central cavities of the trichomes, because they are firmly attached to the epidermis, and the cavities are partially filled with cellular debris. The secondary fluorescence of flavonoids in isolated fresh trichomes was confirmed by CLSM under blue light excitation. Required incubation time in NR was shorter, probably because the isolated trichomes were more accessible to the reagent. Due to the complexity of the identification of flavonoids in their glycoside forms, HPLC analysis of methanolic extracts after acid hydrolysis was performed, revealing the flavonols quercetin, kaempferol and isorhamnetin (3-methylquercetin), the main aglycones in trichomes as well as in leaves. According to our knowledge, within the tribe Alysseae, which includes *Degenia*, flavonoids have been investigated in the genera *Alysum* and *Aurinia* revealing highly glycosylated flavonols, di- and triglycosides of kaempferol, quercetin, and their methyl ethers (Bucar et al. 2005). In *Alyssum alyssoides* kaempferol 3-O-β-D-glucopyranoside (astragalin), kaempferol 3-O-(6″-α-L-rhamnopyranosyl)-β-D-glucopyranoside (nicotiflorin), quercetin 3-O-β-D-glucopyranoside (isoquercetin), quercetin 3-O-β-D-galactopyranoside (hyperoside), isorhamnetin 3-O-β-D-glucopyranoside, isorhamnetin 3-O-β-D-galactopyranoside and isorhamnetin 3-O-(6″-α-L-rhamnopyranosyl)-β-D-glucopyranoside (narcissin) were identified (Tsiftsoglou et al. 2019). Interestingly the concentration of flavonoids, especially quercetin, was several times higher in trichome extracts than in extracts of detrichomized *Degenia* leaf. It is known that simple hairs, i.e. non-glandular trichomes, may contain different flavonoids (Karabourniotis et al. 2020). Methylated kaempferol glycosides were identified in non-glandular trichomes of holm oak leaves (Skaltsa et al. 1994) while kaempferol glycoside derivatives were identified in dendritic trichomes of *Cistus salvifolius* leaves and their ability to absorb UVB and UVA radiation was proven (Tattini et al. 2007). In a study in which *D. velebitica* response to UV stress was investigated, the concentrations of aglycones quercetin, kaempferol and isorhamnetin were significantly higher in UV-exposed plants than in plants grown without UV stress (Stamenković et al. 2015). UV radiation, as well as intense PAR radiation, causes a strong increase in the concentration of flavonoids, specifically of dihydroxy-B-substituted flavonols such as quercetin derivatives which besides their photoprotective role also have a strong antioxidant property (Laoué et al. 2022). Quercetin aglycone and quercetin 3-O-rhamnoside were found in olive leaf trichomes (Liakopoulos et al. 2006). Moreover, the highest density of trichomes and the highest concentration of flavonoids were found in olive leaves on southern exposure and in the outer part of the crown the most exposed to the sun (Liakoura et al. 1997), confirming the role of trichomes in defense against UV radiation in plants with heliomorphic and xeromorphic characteristics. The importance of "hairiness" in the defense against UVB radiation was also demonstrated by the significantly reduced photosynthesis in detrichomized leaves compared to whole leaves in holm oak (Skaltsa et al. 1994) and *Verbascum* species (Manetas 2003).

In *D. velebitica* leaf sections, intense fluorescence after a short incubation in NR, observed by EFM and CLSM, showed the presence of flavonoids in guard cells of stomata while the fluorescence was weaker in the walls and cytoplasm of outer epidermal cells. NR-induced fluorescence was observed in guard cells of *Arabidopsis thaliana* (Brassicaceae) along with the accumulation of ROS that may act as a second messenger during stomatal opening (Watkins et al. 2017). These authors suggested that flavonols, acting as antioxidants, suppress ROS levels in guard cells and thus modulate the dynamics of the stomatal aperture. On transversal sections of *D. velebitica* leaves treated with aqueous ammonia and observed by EFM under UV light excitation, intense green fluorescence of almost all epidermal cells, including vacuoles was visible. It is known that only dihydroxy-B-substituted flavonoids, such as quercetin and isorhamnetin derivatives, after treatment with NR, create adducts that fluoresce intensely when co-excited with blue light (Agati et al. 2009), while glycosides of kaempferol, which is a monohydroxy-B-substituted flavonoid, fluoresce intensely only in alkaline solutions (Hutzler et al. 1998). Therefore, it can be concluded that kaempferol derivatives prevail in the vacuoles of the epidermal cells of *D. velebitica* leaves while quercetin derivates are present in guard cells, walls and cytoplasm of other epidermal cells. In palisade cells, intense fluorescence was limited only to the peripheral parts of the cells in the area of the cell wall, plasma membrane and cytoplasm while the vacuoles did not fluoresce either after incubation in NR, or in alkalized sections. Biosynthesis of flavonoids occurs mainly in the cytoplasm and flavonols are transported and stored in glycosylated form mainly in the vacuoles of guard cells, epidermal and subepidermal cells, but also in the cell walls of the epidermal cells as methylated flavonol glucosides and within the leaf cuticle which optimizes their role as UV-screeners or ROS scavengers (Laoué et al. 2022). Flavonoids are additionally associated to chloroplasts and the cell nucleus where they can reduce the ROS levels and

preserve oxidative damage in various stress conditions (Agati et al. 2020). The above findings indicate that flavonoid accumulation during UV and PAR stress increases the attenuation of excess radiation, but also enhances the antioxidant capacity of the cell to resist oxidative stress.

Conclusion

The localization of flavonoids in the leaf tissue, especially in epidermal cells of *Degenia velebitica*, emphasizes their role as UV filters, but also as non-enzymatic antioxidants. The presence of flavonoids, especially quercetin inside trichomes highlights the important role of this dense covering layer in protecting the sensitive leaf structures of *D. velebitica* from intense radiation, overheating and water loss.

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