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ORIGINAL ARTICLE

Glandular trichomes of the leaves in three *Doronicum* species (Senecioneae, Asteraceae): morphology, histochemistry, and ultrastructure

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Abstract

Two types of glandular tichomes (GTs) develop on the leaves in three *Doronicum* species. The purpose of the work was to establish common and distinctive morphological, anatomical, histochemical, and ultrastructural features of the trichomes. It turned out that differences between types of trichomes are more significant than interspecific ones. For each *Doronicum* species, differences between GTs of two types include the dimensions, intensity of coloration by histochemical dyes, as well as ultrastructural features of the cells. The GTs of the first type are higher than GTs of the second type. Two to three upper cell layers of the first trichomes develop histochemical staining, whereas in the second ones, only apical cells give a positive histochemical reaction. In all trichomes, polysaccharides, polyphenols, and terpenoids are detected. In the GTs of the first type, polysaccharides are synthesized in larger quantity; in the GTs of the second type, synthesis of the secondary metabolites predominates. Main ultrastructural features of the GTs of the first type include proliferation of RER and an activity of Golgi apparatus denoting the synthesis of enzymes and pectin; however, development of SER, diversiform leucoplasts with reticular sheaths, and chloroplasts with peripheral plastid reticulum also demonstrate the synthesis of lipid substances. The ultrastructural characteristics of the second type GTs indicate the primary synthesis of lipid components. Secretion is localized in a periplasmic space of the upper cell layers. The secretory products pass through the cell wall, accumulate in the subcuticular cavity, and rupture it.

Keywords Glandular trichomes · Morphology · Anatomy · Histoxhemistry · Ultrastructure · Secretion

Introduction

Glandular trichomes are external secretory structures localized on aerial vegetative and reproductive organs and accumulate a range of secondary metabolites. These specialized substances function not only as growth regulators (Wagner 1991), but also function to absorb UV radiation (Wollenweber 1993; Karabourniotis and Fasseas 1996). Further, in addition to their role in defending plants against the attack of herbivores and pathogens, these metabolites also act as attractants for pollinators (Heinrich et al. 2002; Appezzato-da-Glória et al. 2012) and play role for fruit dispersal (Werker 2000).

Numerous plants belonging to Asteraceae family are economically important due to their ability to synthesize medicinally important secondary substances. Leaves and flowers of these common medicinal plants such as arnica, burdock, chamomile, coltsfoot, marigold, mugwort, tansy, or yarrow are characterized by the presence of flavonoids (Komissarenko et al. 1988), saponins (Szakiel et al. 2005), sesquiterpene lactones (Kelsey and Shafizadeh 1980; Seaman 1982; Pizza and de Tommasi 1987), as well as coumarins (Gliszczyńska and Brodelius 2012). GTs are found on the surface of all these plants (Schnepf 1969; Figueiredo and Pais 1994; Ciccarelli et al. 2007). Both in the past as well as in recent time, findings confirming a connection between the presence of GTs in Asteraceae species and their involvement in the synthesis of various secondary compounds are reported (Ascensão and Pais 1987; Werker et al. 1994; Urzúa et al. 1997; Spring 2000; Tateo et al. 2001; Heinrich et al. 2002;

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Göpfert et al. 2005; Favi et al. 2008; Gobbo-Neto et al. 2008; Olsson et al. 2009; Appezzato-da-Glória et al. 2012; Muravnik et al. 2016). Most of these plants belong to Anthemideae, Inuleae, Eupatorieae, Heliantheae, and Vernonieae tribes.

Though Senecioneae is the largest tribe in a family, the external secretory structures were described only in species of *Abrotanella* (Swenson 1995), *Doronicum* (Fernández 2003), *Cineraria* (Cron et al. 2006), and *Emilia* (Adedeji and Jewoola 2008). The role of GTs in the synthesis of secondary metabolites was not discussed in any of these publications. Detailed description of morphology, anatomy, histochemistry, and ultrastructure of the glands, as well as the identified composition of the secretory products, was made only on the *Tussilago farfara* GTs (Muravnik et al. 2016). Thus, there is a scientific demand to expand the study in other species with secretory structures that produce the biologically active compounds.

In the publications devoted to the study of the glandular trichomes, the authors have repeatedly noticed that the morphological diversity of the secretory structures is accompanied by differences in the chemical composition of the secretion. Thus, the different modes of secretion were shown in the diverse types of the trichomes in *Salvia aurea* (Serrato-Valenti et al. 1997). The peltate and capitate glands in *Leonotis leonurus* that synthesized the different mono-, di-, and sesquiterpenes had the very different ultrastructure (Ascensão et al. 1997; Ascensão and Pais 1998). In *Calceolaria adscendens*, there is a correlation between the morphology of the trichomes and the class of terpenoids they produce (Sacchetti et al. 1999).

Studies of *Doronicum* species conducted by I. Fernández (Fernández 2003) and in author's laboratory (Kostina and Muravnik 2014) led to only partial agreement. In accordance with the Fernández's opinion, four types of GTs are recognized in several *Doronicum* species. However, the present authors were able to find only three types of GTs in *D. orientale* and *D. macrophyllum*; among them, the first and second types are found on the leaves, and the third type presents on the peduncle and phyllaries. While Fernández's work was devoted to the taxonomic treatment of the *Doronicum* genus on the basis of descriptive morphology of the various organs, including GTs, discovery of trichomes of the different types localized in the different organs was made by the present authors. Therefore, the question arises whether there are any functional differences between trichomes of the different morphological types, and if a connection exists between the morphology of the glandular trichomes and the chemical composition of the secretory products.

Doronicum L. is a genus known as leopard's-banes, containing near 70 species. They are herbaceous perennials native to Europe, southwest Asia, and Siberia. *Doronicum* plants produce yellow, daisy-like flower heads in spring and summer, and due to their bright impressive flowers, *Doronicum* species are often grown in the gardens as ornamental. However, different secondary metabolites are also found to accumulate in this species and thus these plants are widely used in folk medicine. Roots of *D. macrophyllum* contain alkaloids, specifically otosenine, floridanine, doronine (Alieva et al. 1976), and benzfurans (Bohlmann and Abraham 1979). Flowers and leaves include flavonoids apigenin, kaempferol, quercetin, and rutin (Alieva et al. 1979). Roots and the aerial parts of *D. pardalianches* hold numerous known compounds, mainly thymol and a *p*-hydroxy acetophenone derivative (Bohlmann and Abraham 1979). The widespread pentaynene and an unusual sesquiterpene as well as a pyrrolizidine alkaloid were isolated from *D. hungaricum* (Bohlmann and Abraham 1979; Alieva et al. 1976). The essential oils from aerial parts of *D. corsicum* contain sesquiterpene hydrocarbons and oxygenated monoterpenes (Paolini et al. 2007). The question arises whether the synthesis of the above-mentioned secondary compounds occurs in GTs of the aerial organs. In a preliminary study, we have shown that in two *Doronicum* species, the glandular trichomes (GTs) are formed on the leaves, peduncle, and phyllaries (Kostina and Muravnik 2014). Therefore, in this manuscript, we aim the following: (1) to document types of GTs existing on aerial vegetative and reproductive organs in three *Doronicum* species, (2) to establish the possible functions of different types of trichomes based on the composition of substances contained in GTs and detected by histochemical methods, and 3) to determine ultrastructural features of the GT cells participating in the secondary metabolite synthesis. The first part of this work is dedicated to the research of morphology, anatomy, histochemistry, and ultrastructure of the foliar GTs.

Material and methods

Plant species

Doronicum pardalianches is the type species of the genus. It is a perennial herb with a simple straight stem, up to 70–120 cm in height (Fig. 1a). Radical leaves have a long petiole, intermediate stem leaves contain a short petiole with flanges covering a stem, and upper stem leaves are sessile. Inflorescence holds two to six capitulums on the long peduncles. Each capitulum includes the pistillate ray florets and the hermaphrodite disk florets. Phyllaries form two or three rows. Flowering takes place from the end of May to the end of June. *Doronicum orientale* plants are up to 50 cm in height (Fig. 1b). An arrangement of leaves on a stem and of flowers in a capitulum is the same, as in *D. pardalianches*. Flowering occurs from the middle of May to the middle of June. *Doronicum macrophyllum* plants are up to 75–100 cm in

height. At flowering time, they have no radical, but only the middle and upper stem leaves (Fig. 1c). Flowering continues from the end of May to the end of June. The voucher specimens of *D. pardalianches* (LE 01037344), *D. orientale* (LE 01032117), and *D. macrophyllum* (LE 01032118) are deposited in the Herbarium of the Komarov Botanical Institute of the Russian Academy of Sciences, St. Petersburg, Russia. For structural study, leaves of *Doronicum pardalianches* L. were collected in the neighborhood of Vyborg of Leningrad region (60°41.6379′ N, 28°44.4488′ E), whereas the leaves of *D. macrophyllum* Fisch. ex Fisch. and *D. orientale* Hoffm. were collected from the plants cultivated in the botanical garden of the Komarov Botanical Institute in St. Petersburg (59°58.263′ N, 30°19.568′ E). Material was collected in April and May of 2014–2017.

Scanning electron microscopy (SEM)

The small pieces (1 × 5 mm) dissected from young leaves were fixed in a solution of 1.5% (*v*/*v*) glutaraldehyde and 2% (*v*/*v*) paraformaldehyde in 0.1 M cacodylate buffer with 2% sucrose (pH 7.2) for 6 h at 4 °C (Glauert 1980). After that, the pieces were washed three times with cacodylate buffer and post-fixed in cacodylate buffered 1% (*w*/*v*) osmium tetroxide overnight at 4 °C. Then they were dehydrated in 30, 50, and 70% ethanol (each of 10 min) at room temperature, put into isoamyl acetate for 15 min, and critical point–dried with solvent-substituted liquid carbon dioxide in a Hitachi Critical Point Dryer HCP-2 (Hitachi, Japan). The samples attached to SEM mounts and sputter-coated with a thin layer of gold were observed with a JEOL JSM-6390 (JEOL, Japan)

Fig. 1 Plants of three *Doronicum* species. **a** General view of *D. pardalianches* plant. **b** Stem leaves and inflorescences in the form of a capitulum in *D. orientale*. **c** Stem leaves in *D. macrophyllum*

scanning electron microscope at 6 kV accelerating voltage. Digital images were produced using the SEM Control Program associated with this microscope.

Light microscopy (LM) and transmission electron microscopy (TEM)

For light and ultrastructural study, the specimens have been fixed in two ways: (1) as described for the SEM process at 4 °C; (2) in 3% (*v*/*v*) glutaraldehyde with 0.1 M cacodylate buffered 0.1% (*w*/*v*) osmium tetroxide with 2% sucrose (pH 7.2) for 2 h at 4 °C. Then they were transferred to a 3% (*v*/*v*) glutaraldehyde solution without osmium tetroxide in cacodylate buffer with 2% sucrose (pH 7.2) for 14 h at 4 °C. After, the samples were postfixed in cacodylate buffered 1% (*w*/*v*) osmium tetroxide (pH 7.2) for 2 h at 4 °C (Turner et al. 2000). Fixed material was washed three times in cacodylate buffer, dehydrated in 30, 50, and 70% ethanol (each of 10 min), and put into 2% (*w*/*v*) uranyl acetate in 70% alcohol for 3 h at room temperature. Then the samples were subjected to following dehydration procedure in 85 and 96% alcohol (each of 15 min) and two changes in 100% acetone (each of 20 min). The dehydrated fragments were infiltrated and embedded in Epon-Araldite epoxy resin and polymerized for 72 h at 60 °C. Longitudinal and cross semi thin sections of 1–2 μm were prepared with Reichert Ultracut R ultramicrotome (Reichert-Jung GmbH, Heidelberg) by using glass knives. Sections were stained for 5 min in 1% Toluidine blue O in 0.05% (*w*/*v*) borax. Observations of the leaves were carried out with a SteREO Lumar.V12 (Carl Zeiss, Jena, Germany) stereo microscope, equipped with a digital imaging AxioCam MRc5 and AxioVision 4.8 software (Carl Zeiss). Anatomical sections were studied with a light microscope AxioScope.A1, equipped with a filter set 09 (excitation BP 450–490 nm, emission LP 515 nm) and imaging software ZEN 2012 (Carl Zeiss). For autofluorescence detection, a Carl Zeiss filter set 01 was used (excitation BP 365/12 nm, emission LP 397 nm). Ultrathin sections (85 nm) were stained with 2% (*w*/*v*) uranyl acetate for 5 min followed by 2% (*w*/*v*) lead citrate for 5 min. Ultrastructural observations and documentation were carried out with a Hitachi transmission electron microscope H-600 (Hitachi, Tokyo, Japan) at 75 kV. Negatives were processed with a scanner Perfection V750 Pro (Epson, Nagano, Japan).

Histochemistry and fluorescent microscopy (FM)

Cross sections of fresh leaves were made with a razor blade. The main classes of the primary and secondary metabolites in GTs were detected using the following histochemical tests: a 0.05% (*w*/*v*) solution of ruthenium red in water, 5 min (Jensen 1962) or 2% (*w*/*v*) solution of safranin O in water, 5 min (Furst 1979), to detect non cellulosic polysaccharides with acidic groups; 0.05% Toluidine Blue O in water, 5 min (Gutmann 1995), to demonstrate phenols; Nadi test (10% α-naphtol in 40% ethanol and 1% dimethyl para-phenylenediamine chloride in 0.05 M phosphate buffer), 60 min (David and Carde 1964), to demonstrate terpenoids. For detection of autofluorescence, the sections were directly viewed in UV light (365 nm) 5% aluminum trichloride (*w*/*v*) in ethanol, 5 min (Hariri et al. 1991), inducting yellow-green fluorescence at 450 nm was used to demonstrate flavonoids.

Trichome dimensions and statistical analyses

From five to seven pieces of leaves with trichomes from each *Doronicum* species were taken for light and electron microscopy. The diameter of a head and length of a stalk were measured using the image analysis software ImageJ 1.37v (Wayne Rasband, National Institutes of Health, USA). Images of 15 up to 25 mature trichomes of each variance were taken randomly. A value of $p < 0.05$ was considered statistically significant. The trichome length and diameter (± SE) are presented in Fig. 4. Dimensions of two types of the GTs were compared with a One-way Analysis of Variance (ANOVA) using Tukey's multiple comparison test in software StatSoft Statistica 12.5.192.7.

Results

Types and distribution of the glandular and non-glandular trichomes

The leaves of three *Doronicum* species are diverse pubescence in nature, and include both glandular and non-glandular trichomes. GTs are usually biseriate, i.e., they hold two columns of cells developed as a result of one anticlinal division in the initial cell and series of periclinal divisions in the daughter cells. It appears that the divisions in one column did not coordinate with divisions in the second column. The GTs are cylindrical in shape. One or two layers of the upper cells have been described as head, whereas the lower layers are considered as a stalk. Non-glandular trichomes are uniserial or biseriate; they have a pointed tip and differ significantly in length.

Two types of GTs are formed on the stem leaves of *D. pardalianches*. They are located both on adaxial and abaxial side of a lamina (Fig. 2a, b). In GTs of the first type in addition to a head (Fig. 3a, d), there are eight to ten cells that composed the stalk (Fig. 3a). GTs of the second type possess stalks five to six cell layers (Fig. 3g). Besides GTs, large biseriate non-glandular hairs with terminal cells of a conical form, as well short uniseriate hairs, are formed on the lamina (Fig. 2a). In *D. orientale* and *D. macrophyllum*, identical types of the glandular and

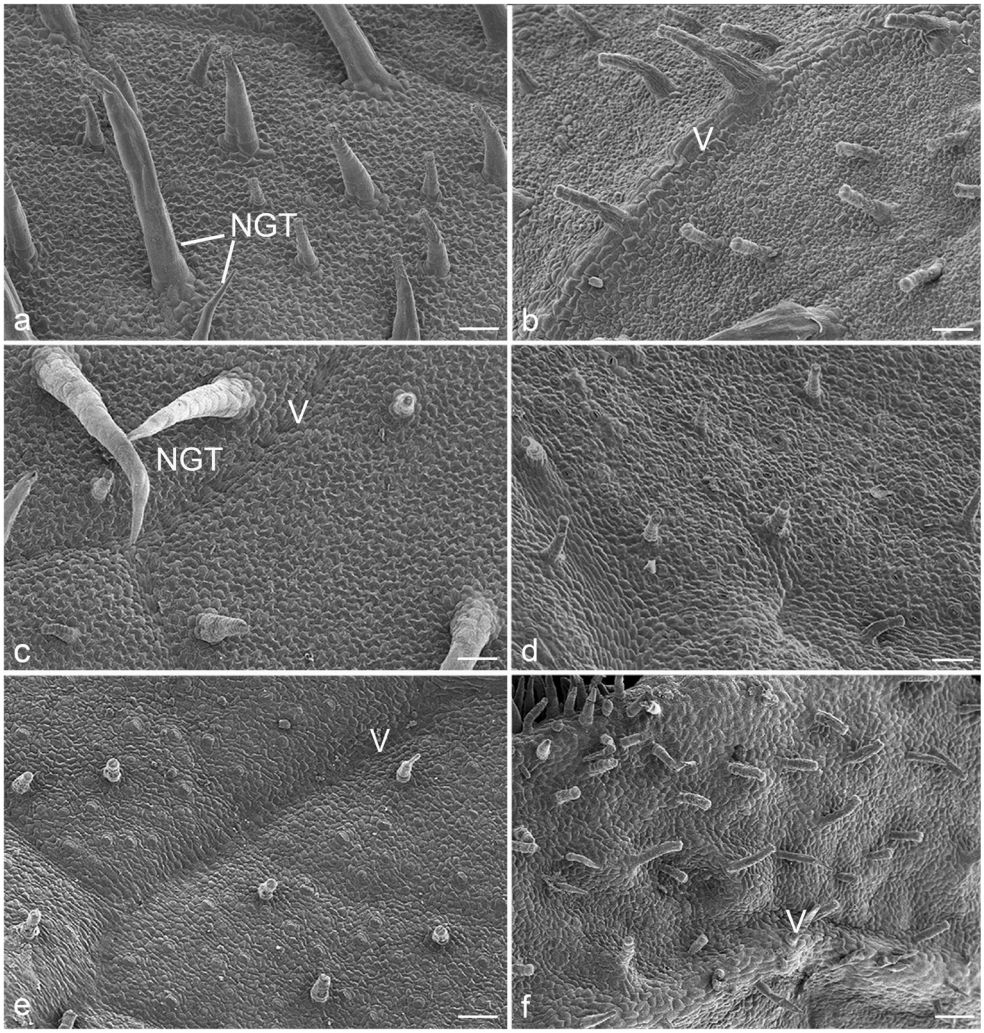

Fig. 2 SEM micrographs of the stem leaves in *Doronicum* species. **a**, **b** Overview of the GTs and non-glandular trichomes on the adaxial (**a**) and abaxial (**b**) side in *D. pardalianches*. **c**, **d** Overview of the GTs and non-glandular trichomes on the adaxial (**c**) and abaxial (**d**) side in *D. orientale*. **e**, **f** Overview of the GTs on the adaxial (**e**) and abaxial (**f**) side in *D. macrophyllum*. Bars 100 μm. NGT non-glandular trichome, V vein

non-glandular trichomes are found (Figs. 2c–f and 3b–l). *Doronicum* species differ in distribution and dimensions of the trichomes (Fig. 4). While in *D. orientale*, GTs of the first type are rare on both sides of the leaf lamina, in *D. macrophyllum*, they are more common on the abaxial surface than on the adaxial one; and GTs of the second type are formed on the abaxial side only. The length of each type of the GTs varies significantly between three species. Furthermore, in each species, the length of the GTs of the first type is reliably larger than this characteristic in the GTs of the second type (Fig. 4).

One of the features of the mature GTs of both types in *Doronicum* species is the development of a subcuticular cavity in the head (Fig. 3), where accumulation of the secretion takes place. Initially, secretion has been shown to be precipitated between external cell wall and cuticle forming the small eminences (Fig. 3a). Subsequently, they join and create a total subcuticular cavity in the form of a dome. The cuticle can break (Fig. 3d–l) to release the secretion to the outside.

Anatomy of the glandular trichomes

On the longitudinal sections of GTs of the first type in all studied species (Fig. 5a–c), four to six upper cell layers contain dense cytoplasm, all lower layers form large vacuoles. In GTs of the second type, the upper layers of cells look the same as in trichomes of the first type, whereas one or two lower layers of a stalk are vacuolated (Fig. 5d–f).

Histochemistry and FM of the glandular trichomes

Fresh unstained sections of GTs of the first and second types have a light-green color or are colorless (Fig. 6a, f). Various substances were found in the trichomes by histochemical tests and treatment of fluorescent reagents. The presence of lipids was demonstrated by green autofluorescence in the head cells of both trichome types (Fig. 6b). Fluorochrome aluminum chloride induced the yellow-green fluorescence of flavonoids in the head cells (Fig. 6g). Red autofluorescence, characteristic of chlorophyll of chloroplasts, was seen in the stalk cells

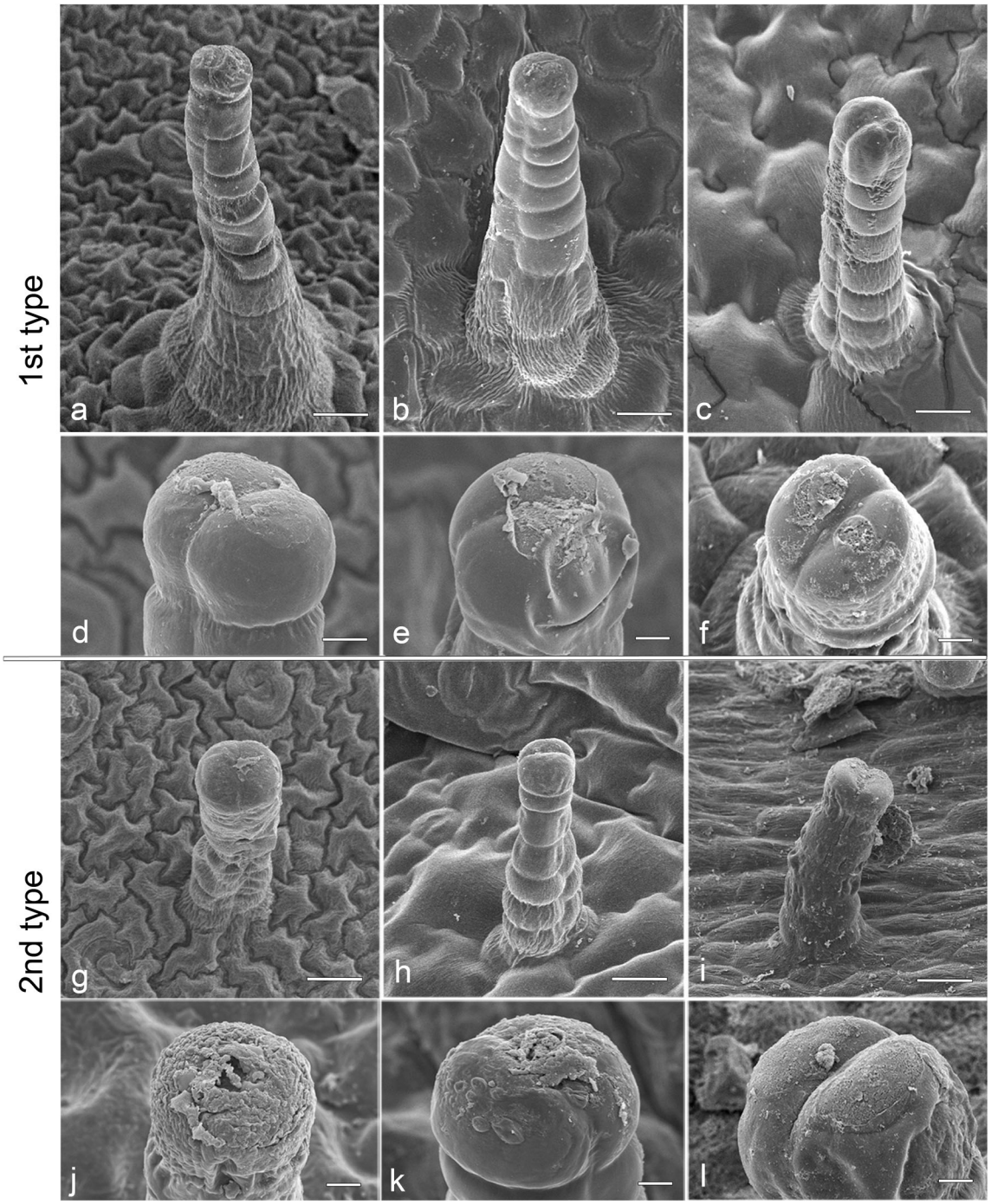

Fig. 3 SEM micrographs of the foliar GTs of two types in *Doronicum* species. **a**–**c** GTs of the first type, abaxial side. **d**–**f** Heads of the GTs of the first type. Ruptures of a cuticle are obvious. **g**–**i** GTs of the second type; abaxial side (**g**, **h**), adaxial side (**i**). **j**–**l** Heads of the GTs of the second type with a broken cuticle. **a**, **d**, **g**, **j** *D. pardalianches*, **b**, **e**, **h**, **k** *D. orientale*, **c**, **f**, **i**, **l** *D. macrophyllum*. Bars 20 μm (**a**–**c**, **g**–**i**), 5 μm (**d**–**f**, **j**–**l**)

(Fig. 6b, g). Acidic polysaccharides were found in the head cells and in upper stalk cells of GTs of the first type (Fig. 6c) as well as in the apical cell and on the head surface in the form of a drop of slime in GTs of the second type (Fig. 6h). Accumulation of phenols in the head cells was identified by dark-blue or turquoise color (Fig. 6d, i) using toluidine blue. Terpenoids were found with Nadi reagent as violet coloration in the apical layer of the head, particularly in subcuticular cavity (Fig. 6e, j). In GTs of the first type, the upper stalk cells were also colored (Fig. 6e). GTs of the each type did not demonstrate any substantial differences between *Doronicum* species.

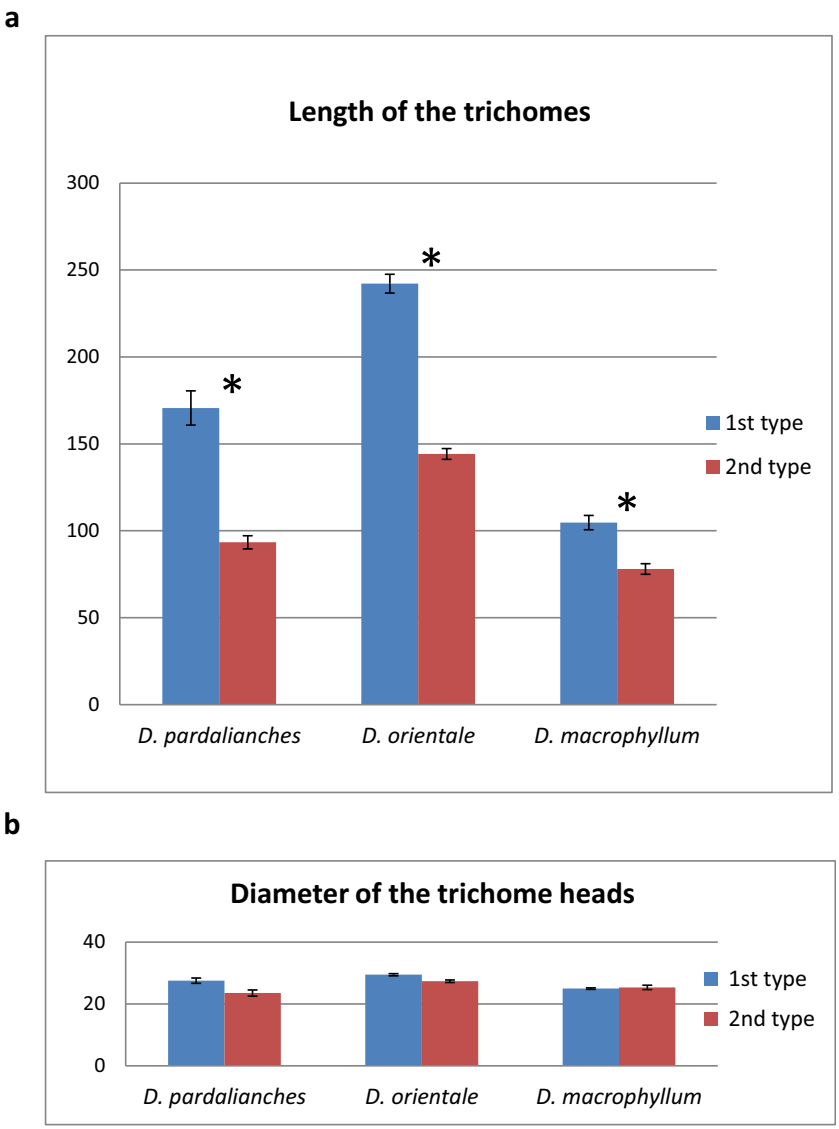

Fig. 4 Dimensions of the GTs of two types in *Doronicum* species. **a** Length of the trichomes. **b** Diameter of the trichome head. Values are means ± SE, $n = 15–25$. Statistically significant differences between the trichomes of the first and second type in their length exist in each *Doronicum* species at $p < 0.05$, as is determined by a Tukey HSD test; they are indicated with *

Ultrastructure of the glandular trichomes

GTs of the first type in three *Doronicum* species have the similar ultrastructural features (Fig. 7a). Each of three upper layers of the cells possesses some specific characteristics. In the head cells, a large rounded or amoeboid-shaped nucleus occupies a central position and the cytoplasm contains numerous organelles and free ribosomes. The main organelle of all apical cells is a smooth endoplasmic reticulum (SER). It runs through the cytosol as dense network (Fig. 7b); many tubules are dilated and become similar to small transparent vacuoles (Fig. 7a, c). In the elements of SER, the black inclusions are sometimes observed (Fig. 7c); if tubules are situated in the peripheral cytoplasm and contact with the plasma membrane, the black inclusions appear in the periplasmic space (Fig. 7d). In the cells lying under apical, SER is developed in the same way as in the apical cells. In the head cells, besides tubules of SER, there is rough endoplasmic reticulum (RER); at times, it forms the large aggregates of cisterns (Fig. 7e). The activity of the Golgi apparatus is moderate. The Golgi bodies consisting of five to seven cisterns (Fig. 7f) produce small vesicles delimited by a smooth membrane and coated vesicles. Dimensions and structure of the plastids are changing in the direction from the apical to lower cells. The small round leucoplasts are typical for the apical cells (Fig. 7g); they contain single lamellae and peripheral plastid reticulum. In the cells of the second and third layers, leucoplasts are larger; they frequently contain plastoglobuli (Fig. 7h); in addition, the osmiophilic inclusions appear in the plastid lamellae. Leucoplasts of *D. orientale* have a very diverse form. As a rule, short elements of the endoplasmic reticulum are situated near the outer membrane of the leucoplast envelope (Fig. 7h). In the cells of a stalk, leucoplasts are absent. There are many chloroplasts in the cells of the second and third layer (Fig. 7i).

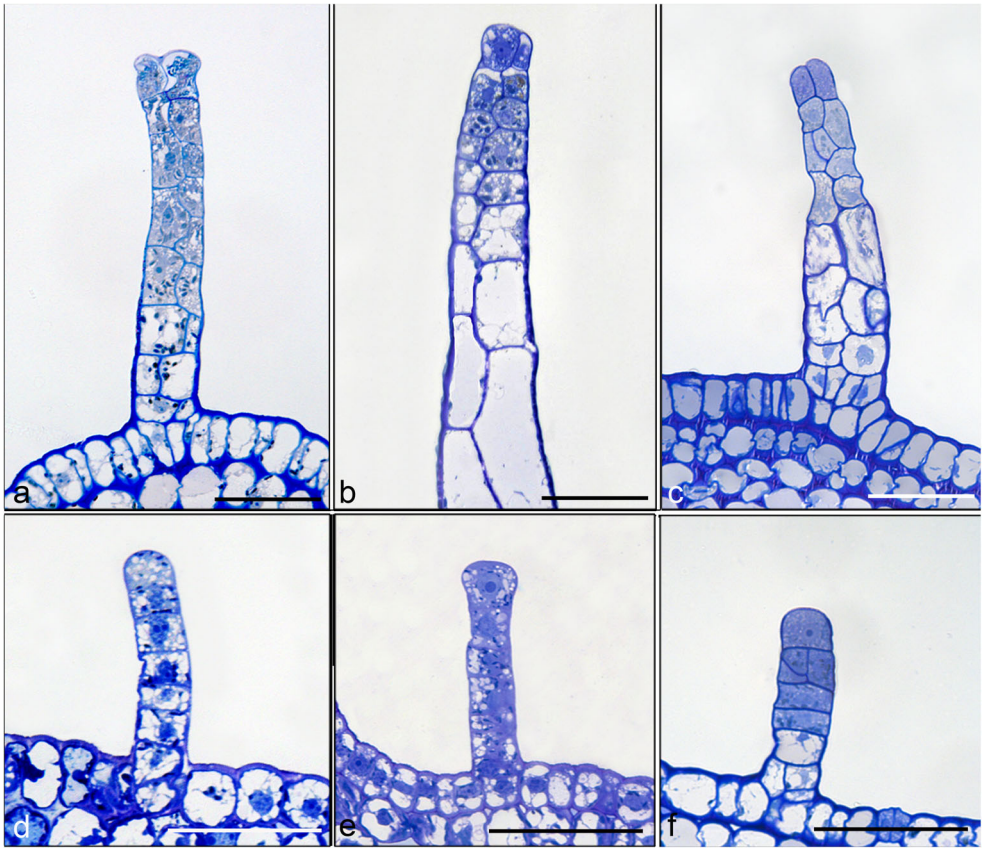

Fig. 5 LM micrographs of the longitudinal sections of the GTs in *Doronicum* species. **a**–**c** GTs of the first type. **d**–**f** GTs of the second type. **a**, **d** *D. pardalianches*, **b**, **e** *D. orientale*, **c**, **f** *D. macrophyllum*. Bars 50 μm

Often, chloroplasts have an irregular form, including cup-shaped invaginations; in their stroma, a peripheral plastid reticulum is found; chloroplasts are surrounded by reticular cisterns (Fig. 7i). Mitochondria have an orthodox structure (Fig. 7g). The same black deposition, as in the external cell wall, is located along the anticlinal and periclinal walls in the second and third layer of GTs. The vacuoles in these cells are larger than in the apical cells and have different contents (Fig. 7a). In GTs of *D. pardalianches*, in contrast to *D. orientale* and *D. macrophyllum*, vacuoles are felt with dark contents.

In GTs of the second type (Fig. 8a), SER is intensively developed in all upper cell layers (Fig. 8b), whereas RER is represented by rare and short cisterns (Fig. 8c). Golgi bodies detach the few vesicles with smooth membrane (Fig. 8d); *trans*-Golgi reticulum (TGR) with the coated vesicles is found near them (Fig. 8e). In the apical cells of the trichomes of the second type just like in the trichomes of the first type, the small leucoplasts are formed, whereas in the second and third layer, there are both leucoplasts and chloroplasts. Leucoplasts contain numerous plastoglobuli, lamellae, and peripheral plastid reticulum (Fig. 8f). In chloroplasts, thylakoids occupy only a small part, the remaining part is filled with a peripheral plastid reticulum. Some chloroplasts have cup-shaped invaginations (Fig. 8g). Reticular sheaths are typical both for leucoplasts and chloroplasts. Onset of secretion is accompanied by the appearance of the dark contents in the lamellae and reticular sheaths of the leucoplasts as well as in the periplasmic space. Quantity of secretion in the GTs of the second type is larger than in the GTs of the first type (Fig. 8h). In the cytosol, there are gray lipid drops and large black inclusions of reserve substances (Fig. 8i).

During the development of the GTs of the first and second type, the structure of the external cell wall and cuticle in the apical cells significantly changes (Fig. 9). While, the cell wall is more or less homogeneous, the cuticle consists of a thin layer of the cuticle proper and cutinized layer of the cell wall. The cutinized layer contains amorphous globules (Fig. 9a), dendritic structures (Fig. 9b), and light interfibrillar lamellae (Fig. 9c). The interfibrillar lamellae are easily identified in GTs of *D. pardalianches* and *D. macrophyllum*, whereas the dendritic structures are better seen in *D. orientale*. Before onset of secretion, the periplasmic space in the head cells is almost absent (Fig. 9a–c). It starts when an osmiophilic deposition is revealed outside the plasma membrane (Fig. 9d–f). Then that an amorphous light-colored secretion is accumulated in the cutinized layer of the cell wall (Fig. 9d–f). The structure of the cutinized layer looks loose. Single small sites containing light-colored secretion (Fig. 9d, thin black arrows) fuse with each other and form large light sites (Fig. 9d–h, thick black arrow). Then in the cutinized layer, the thin light strands arise; they are located perpendicular to the cell wall towards the cuticle proper (Fig. 9g-i). The more the secretion

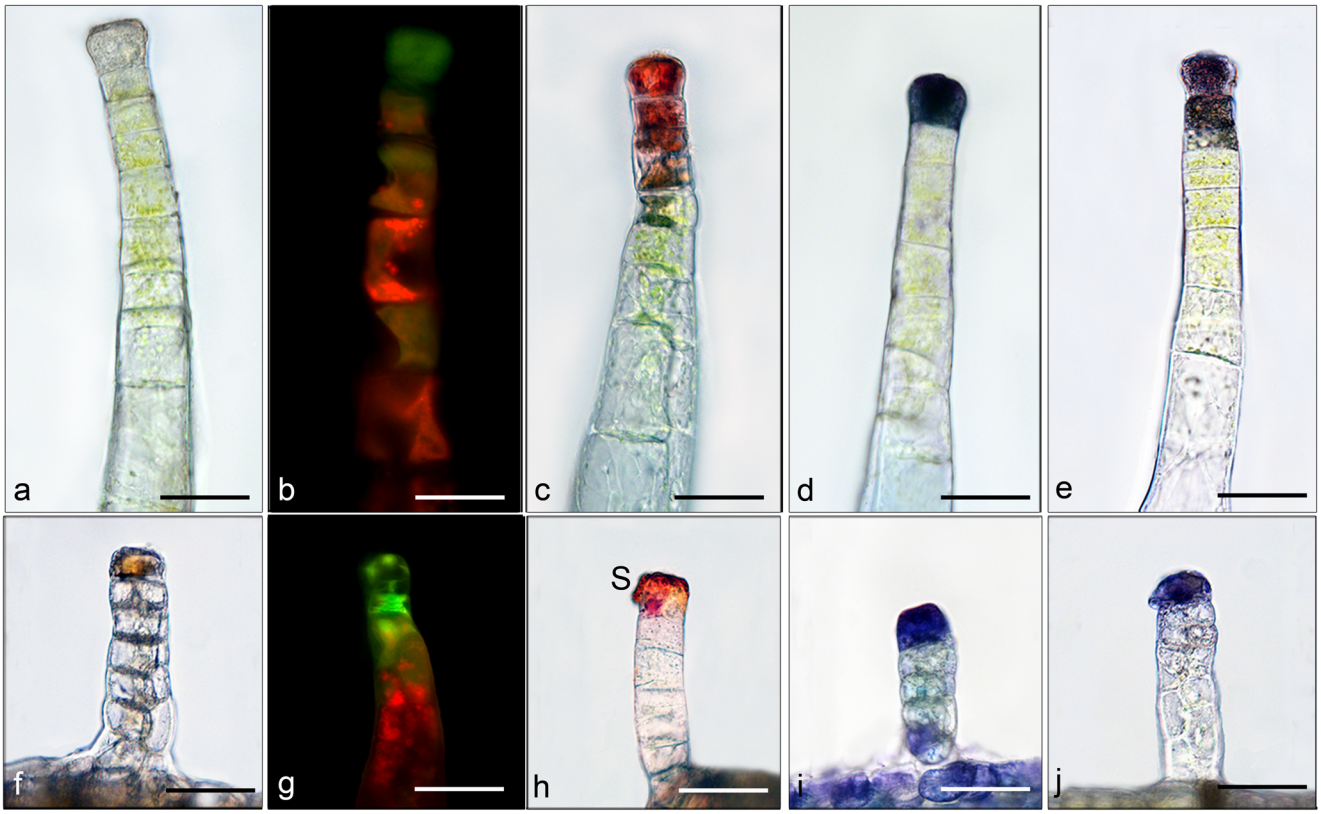

Fig. 6 LM micrographs showing reaction to histochemical tests of the GTs of two types in *Doronicum* species. **a**–**e** *D. pardalianches*, **f**–**j** *D. orientale*. **a**, **f** Fresh sections of the GTs without dye have a native colorless (**a**) or yellowish (**f**) head and the greenish or colorless stalk cells. **b**, **g** Induction of green autofluorescence (**b**) and the yellow-green fluorescence after treatment with aluminum chloride (**g**) of flavonoids is a characteristic in the head cells in UV light; red autofluorescence of chloroplasts is seen in the stalk cells. **c**, **h** Acid polysaccharides are detected in the head cells as red coloration after treatment by ruthenium red (**c**) or safranin O (**h**). S secretion. **d**, **i** Phenols are found in the head cells as blue coloration after toluidine blue O. **e**, **j** Terpenoids give dark-blue color of the head cells after staining with NADI reagent. Bars 50 μm

accumulates, the longer the strands become. As a result, a subcuticular cavity appears and grows (Fig. 9g); eventually, a cuticle ruptures and the secretion is released. This scenario was observed in the GTs of the first and second type in three studied *Doronicum* species.

Discussion

GTs of two types were found on the leaf surface in each of three *Doronicum* species. Although trichomes are predominantly cylindrical in shape, they can be conditionally subdivided into head and stalk. Trichomes with small two-celled head on a high stalk belong to the first type; trichomes on a short stalk belong to the second type. Dimensions of the GTs of both types reliably differ; the highest GTs are found in *D. orientale*. The formation of two columns of the cells, i.e., biseriate structure of the GTs, is typical for Asteraceae species; it is described in *Arctium lappa* (Schnepf 1969), *Artemisia annua* (Duke and Paul 1993), *Helichrysum aureonitens* (Afolayan and Meyer 1995), *Chaptalia integerrina* (Castro et al. 1997); *Matricaria chamomilla* (Andreucci et al. 2008), *Vernonia galamensis* (Favi et al. 2008), *Helianthus annuus* (Amrehn et al. 2014); and in others plants.

In *Doronicum*, the mature GTs of the first and second types are able to synthesize secretion that is accumulated in the cytoplasm or subcuticular cavity. In two types of the GTs, the identical histochemical tests were made and the chemical content of secretion was detected. It turned out that acidic polysaccharides, polyphenols, and terpenes are identified in trichomes. In spite of the presence of the same classes of substances, there are some differences between two types, which are expressed in localization and quantity of the reaction products. In the GTs of the first type, positive reaction to pectin and secondary compounds is visible in the head cells and in one to two upper layers of a stalk, whereas in the GTs of the second type, only the head cells are colored (Fig. 6c, h). In the GTs of the second type, the fluorescence of flavonoids is higher than in the GTs of the first type (Fig. 6b, g). Dark yellow or green fluorescence is determined by structural type of the flavonoids (Combrinck 2006). Different chemical compounds localize in the different compartments of each trichome type. Acid polysaccharides are visible in the cytoplasm of the upper cell layers and in the secretory drop on the surface

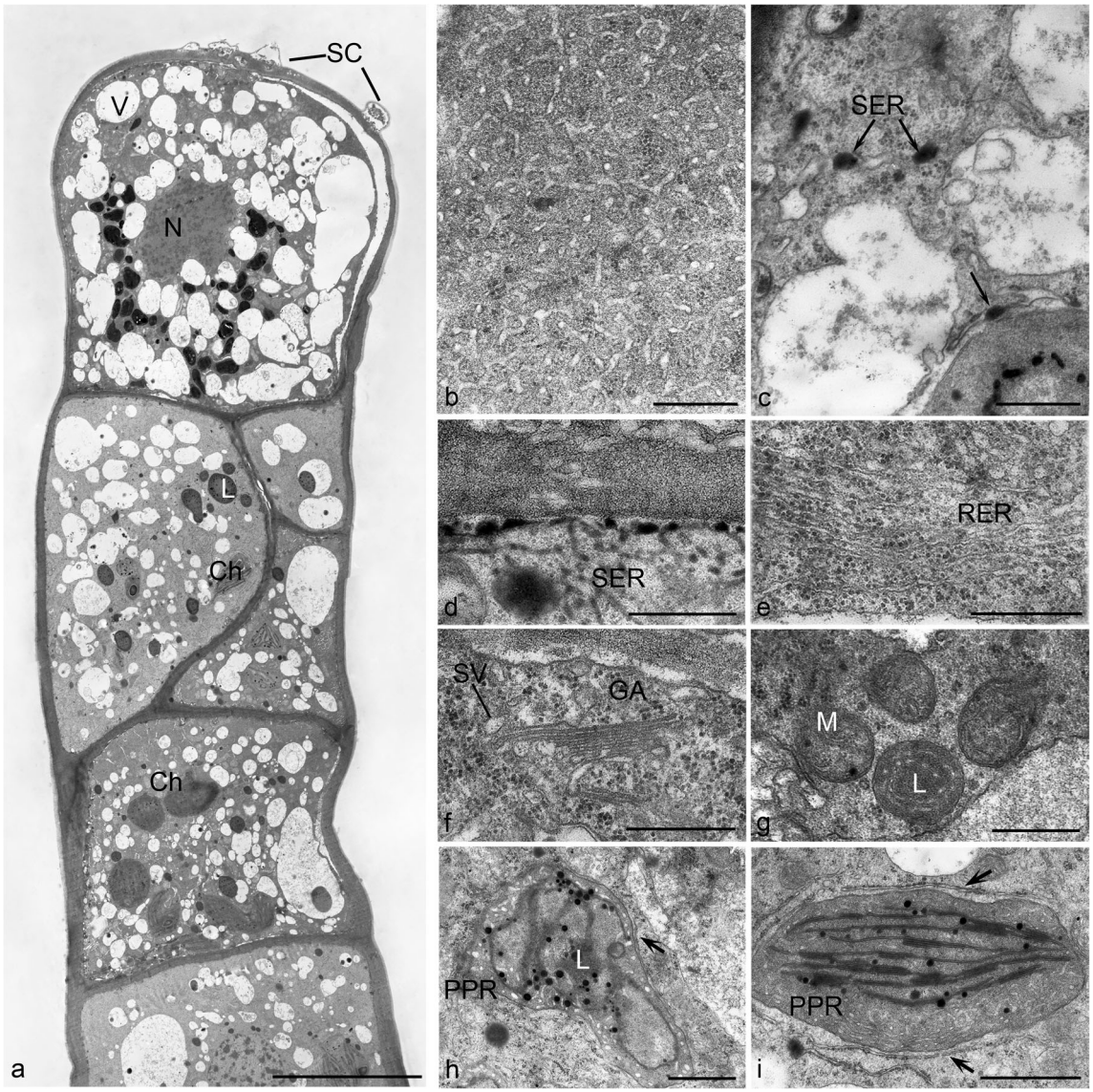

Fig. 7 Ultrastructure of the foliar GTs of the first type in *Doronicum* species. **a**, **b**, **i** *D. orientale*, **c**, **e**, **h** *D. macrophyllum*, **d**, **f**, **g** *D. pardalianches*. **a** Longitudinal section of the four upper cell layers. The terminal cell contains numerous small vacuoles (V). The subcuticular cavity (SC) begins to form over the outer cell wall. Ch chloroplast, L leucoplast, N nucleus. **b** Tubules of the smooth endoplasmic reticulum (SER) penetrate the cytoplasm. **c** Some elements of SER contain the black inclusions (arrows). **d** Appearance of the osmiophilic contents in the periplasmic space. **e** Aggregates of cisterns of the rough endoplasmic reticulum (RER) are met in the apical cells. **f** Golgi stack with smooth membrane vesicles (SV) in the apical cells. **g** The small oval leucoplasts are in the apical cells, M mitochondrion. **h** In the cells of the second layer, there are large leucoplasts of various forms with peripheral plastid reticulum (PPR), lamellae, and plastoglobuli. Elements of endoplasmic reticulum surround the plastid envelope (arrow). **i** Chloroplasts there are in all lower cell layers. They are also surrounded with reticular sheaths (arrow). **a–c**, **i** Fixation with glutaraldehyde and paraformaldehyde. **d–h** Fixation with glutaraldehyde and osmium tetroxide. Bars 10 μm (**a**), 1 μm (**h**, **i**), 0.5 μm (**b–g**)

of a head, terpenes are in the cytoplasm and subcuticular cavity of the GTs, and phenolic substances are distinguished only in the cytoplasm.

Previously, acidic polysaccharides were demonstrated in GTs of some Asteraceae species, belonging to different tribes, namely, in *Inula viscosa* from *Inuleae* (Werker and Fahn 1981), *Artemisia campestris* from *Anthemideae* (Ascensão and Pais 1987), *Stevia rebaudiana* from *Eupatorieae* (Monteiro et al. 2001), and *Tussilago farfara* from *Senecioneae* (Muravnik et al. 2016). Phenols were shown in the glandular hairs of *I. viscosa* (Nikolakaki and Christodoulakis 2004) and *Matricaria chamomilla* (Andreucci et al. 2008). Flavonoids are found in the trichomes of *Sigesbeckia jorullensis* (Heinrich et al. 2002) and *T. farfara* (Muravnik et al. 2016). Terpenes are revealed in the GTs in *S. rebaudiana* (Tateo et al. 2001), *Santolina ligustica* (Pagni et al. 2003), *M. chamomilla* (Andreucci et al. 2008), as well as in two *Chrysolaena* species (Appezzato-da-Glória et al. 2012).

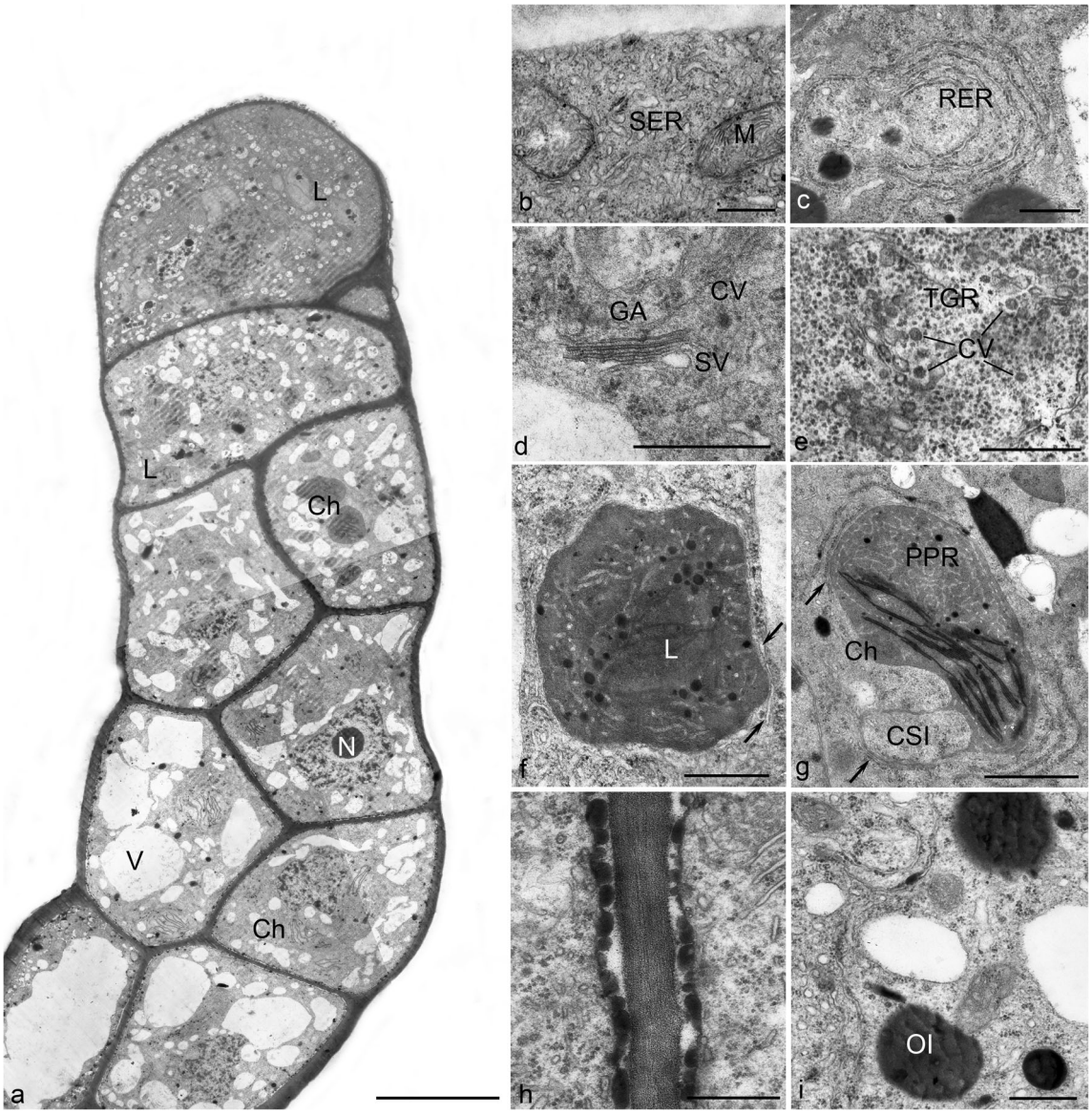

Fig. 8 Ultrastructure of the foliar GTs of the second type in *Doronicum* species. **a**, **c**, **g**, **i** *D. orientale*, **b**, **e** *D. pardalianches*, **d**, **f**, **h** *D. macrophyllum*. **a** Longitudinal section of the five cell layers. Ch chloroplast, L leucoplast, N nucleus, V vacuole. **b** Tubules of the smooth endoplasmic reticulum (SER) in the peripheral cytoplasm of the apical cell. M mitochondrion. **c** Accumulation of some cisterns of the rough endoplasmic reticulum (RER) in a cell of the second layer. **d** Golgi apparatus forms both vesicles with smooth membrane (SV) and coated vesicles (CV) in the cell of the second layer. **e** Trans-Golgi reticulum (TGR) with coated vesicles in the apical cell. **f** Leucoplast in the cell of the second layer contains numerous lamellae and plastoglobuli. Cisterns of RER lie near the plastid envelope (arrows). **g** Chloroplast forms cup-shaped invagination (CSI). Chloroplast envelope is surrounded with reticular sheaths (arrows). **h** Anticlinal wall in the third layer. Periplasmic space contains black secretion. **i** Osmiofilic inclusions (OI) there are in the cytoplasm of third layer. **a**, **c**, **g**–**i** Fixation with glutaraldehyde and paraformaldehyde. **b**, **d**–**f** Fixation with glutaraldehyde and osmium tetroxide. Bars 10 μm (**a**), 1 μm (**g**), 0.5 μm (**b**–**f, h**–**i**)

The difference between the two types of GTs expressed in their dimensions and histochemical reactions suggests a different functional specialization of these secretory structures. To examine this assumption, an ultrastructure of the trichomes was studied in each of three *Doronicum* species. Obviously, several upper layers of the cells with dense cytoplasm are involved in the consistent stages of secretion biosynthesis. In two upper cell layers, RER cisterns are usually found. In the trichomes of the first type, proliferation of the RER essentially exceeds that in the trichomes of the second type (Figs. 7e and 8c). Simultaneously, in the GTs of the first type, Golgi apparatus produces transparent vesicles with smooth membrane, which usually carry polysaccharides and then throw them out by granulocrine, or exocytose way (Hawes and Satiat-Jeunemaitre 1996; Andreeva et al. 1998). These ultrastructural features denote on synthesis and transport of the primary

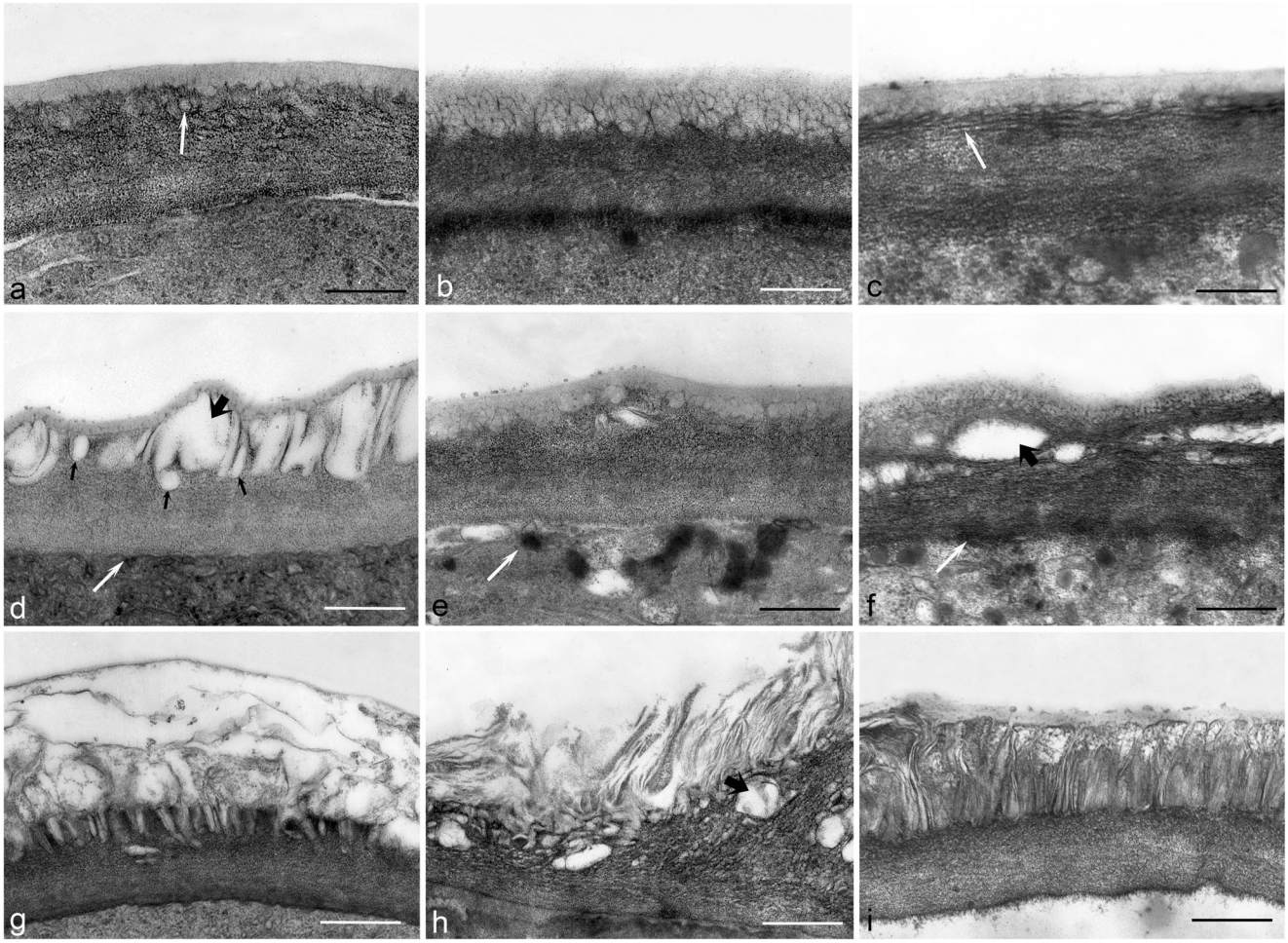

Fig. 9 Ultrastructure of the external cell wall and cuticle in the GTs of two types in *Doronicum* species. **a**, **d**, **g** *D. pardalianches*, **b**, **e**, **h** *D. orientale*, **c**, **f**, **i** *D. macrophyllum*. **a**–**c** The amorphous globules (**a**), dendritic structures (**b**), and light short lamellae (**c**) are seen in the cutinized layer before secretion. **d**–**f** Osmiophilic deposition appears in a periplasmic space (white arrows) after beginning of secretion. The small light-colored secretory globules (**d**, thin black arrows) occur in the cutinized layer and fuse with each other (**d**, **f**, **h**, thick black arrow). **g**–**i** Formation of the numerous microchannels in cutinized layer. Abundant secretion there is in subcuticular space (**g**). Bars 0.5 μm

metabolites, particularly, pectin components of secretion. Thereby, ultrastructural data confirm the results of histochemistry about dominance of the pectins in the cells of GTs of the first type as compared with the GTs of the second type. In GTs of the second type, the activity of the Golgi apparatus is predominately aimed at the production of the hydrolytic enzymes that is confirmed by the appearance of the *trans*-Golgi reticulum with coated vesicles in the upper cell layers. Just with coated vesicles, it is common to associate the transfer of acid hydrolases through the plasma membrane (Beevers 1996; Sanderfoot and Raikhel 1999). The hydrolytic enzymes are necessary for loosening of the apical cell wall to facilitate transfer of the secretory products (Ascensão et al. 1997; Turner et al. 2000; Schönherr 2006).

In all cells of the upper layers of the GTs, SER is the main organelle. Commonly, it appears in the glands, in which lipids, including polyphenols and terpenes, are synthesized (Schopker et al. 1995; Turner et al. 2000; Vassilyev 2000; Machado et al. 2006). In both types of GTs of *Doronicum* species, the black inclusions are revealed within the reticular tubes with the smooth membrane, as it was shown in GTs of *Sigesbeckia jorullensis* (Heinrich et al. 2010) and *Tussilago farfara* (Muravnik et al. 2016). The similar deposition accumulated in a periplasmic space indicates secretion of the secondary metabolites from the cytoplasm. Deposit is not only in the apical cell layer but also outside plasma membrane of the cells of two underlying layers. In the GTs of the second type, there is a larger amount of the black globules than in the GTs of the first type (Figs. 7d and 8h).

The leucoplasts belong to the necessary organelles participating in biosynthesis of terpenes and phenylpropanoids (Duke and Paul 1993; Ascensão and Pais 1998; Turner and Croteau 2004; Muravnik and Shavarda 2012). In *Doronicum* species, there are small oval or ring leucoplasts in the head cells. Their internal membrane system is weakly developed. However, in the cells of the second and third layer, leucoplasts

are larger and more diverse; they often form cup-shaped invaginations. In the GTs of the second type, a stroma of the leucoplasts has a larger electron density than in the GTs of the first type (Figs. 7h and 8f). Around the plastid envelope, there is a reticular sheath as well as in other lipid glands (Ascensão and Pais 1998; Turner et al. 2000; Heinrich et al. 2010).

In addition to leucoplasts, in GTs of three studied species, there are chloroplasts, just like in another Asteraceae (Vermeer and Peterson 1979; Werker and Fahn 1981; Figueiredo and Pais 1994; Afolayan and Meyer 1995; Favi et al. 2008; Heinrich et al. 2010). The chloroplasts have some structural features, which distinguish them from the mesophyll chloroplasts. They include the diverse form of plastids, the abundant tubes of the peripheral plastid reticulum, and occurrence of the reticular sheath near an external membrane. Evidently, the chloroplasts in *Doronicum* species combine a structure and functions of both typical photosynthetic plastids and of leucoplasts participating in the synthesis of the secondary substances. Above-mentioned structural characteristics are mostly expressed in the chloroplasts of the GTs of the second type (Figs. 7i and 8g).

Until now, the mechanisms for removing of the lipid compounds from cytoplasm have remained controversial. As the processes of biosynthesis of phenols and terpenoids involve different intracellular compartments, it is obviously, that transfer of the recently synthesized substances should also be realized by the different ways. Thus, phenylpropanoids and flavonoids are synthesized by the enzyme complexes that locate on the cytosolic face of the endoplasmic reticulum (Winkel-Shirley 1999). In our opinion, being in the cytosol, phenolic compounds pass through the lipid bilayer of the plasma membrane by the eccrine pathway. They are also sequestered in the vacuoles through a glutathione pump in the tonoplast (Alfenito et al. 1998). On the other hand, enzymes of the monoterpene biosynthesis are found within of leucoplasts and endoplasmic reticulum (Turner and Croteau 2004). In order to appear in the cell wall, monoterpenes must overcome two boundary membranes, the endoplasmic reticulum and plasma membrane. For this reason, monoterpenes can be carried outside as a result of the fusion of the reticular and plasma membrane (Skubatz and Kunkel 1999) or through temporary contacts between endoplasmic reticulum and plasma membrane (Vassilyev 2000). Although, in the GTs of *Doronicum* species, the tubes of SER are often located near the plasma membrane, we have not been able to see a picture of exocytosis of the reticulum content to periplasmic space. Thereby, we consider a point of view about the temporary contacts between membranes more appropriate.

After overcoming the plasma membrane, the secretion synthesized in the GTs moves along the cell wall and appears beneath the cuticle. Further ways depend on the chemical content of the secretion. It is known that hydrophilic substances, particularly polysaccharides, move across the cuticle along the branched dendritic structures, called otherwise fibrillary network, reticulate cuticle, or microchannels (Holloway 1982; Lyshede 1982). The strands found in this work, which come from the cell wall in the direction of the cuticle proper and orient perpendicular to the surface of the outer wall, were consider as microchannels. They are formed in the GTs of *Doronicum* plants to facilitate secretion of the hydrated compounds as it was shown in the nectary cells (Koteyeva 2005; Wist and Davis 2006; Kowalkowska et al. 2017). For lipophilic substances, there is a parallel pathway, including cutin and amorphous waxes (Schreiber 2005; Schönherr 2006). According to the authors, the permeability of the cuticle for lipids is increased with increasing solubility of the hydrophobic substances in these polymers. As for the GTs of *Doronicum*, the disappearance of the osmiophilic deposition in the periplasmic space is probably explained by the solubility of lipid secretion in the cutin. The formation of a subcuticular cavity is due to accumulation of a large amount of secretion.

As it is seen from the ultrastructural characteristics of the GTs in three *Doronicum* species, they differ from each other insignificantly. The ultrastructural features of the external cell wall really have some species-specific characters expressing in the occurrence of amorphous globules, dendritic structures, and interfibrillar lamellae; however, these features apparently do not affect the functioning of the GTs. The differences between the types of GTs are more significant; they are determined by the biological role that each type of the GTs plays.

Conclusions

GTs of the two types are formed on the leaves in three *Doronicum* species. Trichomes of the first type contain a two-celled head and high stalk from eight to ten cell layers; trichomes of the second type have the same head and a short stalk from five to six layers. Histochemical tests and fluorescent microscopy revealed acid polysaccharides, polyphenols, flavonoids, and terpenoids in the GTs of both types. Based on the morphology, ultrastructure, and chemical content of secretion, it can be concluded that three *Doronicum* species have similar characteristics within each morphological type of the trichomes. Small diversity refers to the dimensions and distribution of the GTs on the leaf surface. At the same time, there are evident differences between two types of the GTs which are due to the diverse composition of the substances they synthesize. Since GTs of the first type in a greater degree synthesize acid polysaccharides than GTs of the second type, they likely protect the growing shoots from mechanical damage during germination. GTs of the second type, synthesizing mainly flavonoids and terpenoids, are able to defend the young leaves from pathogens and herbivores.

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Author contribution statement LEM conceived and coordinated all experiments, analyzed data, and wrote the manuscript; OVK and AAM contributed to histochemistry and microscopy.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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