Light, Conventional and Environmental Scanning Electron Microscopy of the Trichomes of Cucurbita pepo subsp. pepo var. styriaca and Histochemistry of Glandular Secretory Products

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INTRODUCTION

Cucurbita pepo var. styriaca belongs to the family Cucurbitaceae, which contains approx. 114 genera and 500 species. They are warm-season annuals growing in hot and humid weather. At the beginning of the 20th century a mutation introduced the variation Cucurbita pepo L. subsp. pepo var. styriaca (Greb.) (Teppner, 2000). Most species of Cucurbita plants possess glandular and non-glandular trichomes which are a common anatomical feature of higher plants.

The botanical literature contains more than 300 descriptions of trichome types in order to characterize their great variation. The trichome appendages arise from a series of anticlinal and periclinal divisions of epidermal cells to form specialized trichomes that function as glandular or non-glandular trichomes. Such integral elements of the plant surface, which are all outer growths of the epidermis, are termed as trichomes (Esau, 1965; Johanson, 1975; Fahn, 1979; Werker, 2000). Differences in the habitus of plant trichomes are used in plant classification, i.e. they are taxonomically very useful. Functionally, trichomes protect the plants from herbivores, from heat and sunlight (Croteau, 1977; Werker, 1993; Duke, 1994). Further, they control leaf temperature as well as water loss. Glandular trichomes produce various substances, which are stored at the plant surface (Wagner, 1991). They can be found as unicellular, multicellular and stalked structures. However, the majority consists of a foot, a stalk and a head region. Most morphological and histochemical investigations have been made on species of Asteraceae (Monteiro et al., 2001) or Lamiaceae (Ascensao et al., 1999; Bisio et al., 1999).

This paper presents the results of a morphological and histochemical study of different trichome types on leaves of Styrian oil pumpkin (Cucurbita pepo var. styriaca) with regard to the ontogenetical development by using light-and scanning electron microscopy. The four ontogenetical independent glandular- and non-glandular trichomes and the bristle hairs of Styrian oil pumpkin can be found on both leaf sides and they can be classified considering their morphology, distribution and histochemistry. In a previous paper, four different trichome types of glandular and non-glandular trichomes (types I, II, III and IV) were characterized: type I is the ‘characteristically’ short-stalked Cucurbita type with four cells in the head region; type II is a...
long-stalked, uniseriated trichome with a two-celled glandular head; type IV, the ‘stipitate-capitate’ type, is the biggest and is also a glandular emergence; and type III is the ‘columnar-digit’ trichome (Kolb and Müller, 2003).

The dynamic process of secretory release by intact, undamaged mature trichomes can be observed using the environmental scanning microscopy (ESEM). The ESEM mode enables the investigation of unprepared, water-containing material with the benefits of conventional scanning electron microscopy (CSEM) (depth of focus and three-dimensional imaging of surfaces with high resolution) (Danilatos, 1993). In the ESEM mode it is possible to maintain a rather high humidity (pressure from 10 to 0 torr) in the sample chamber while simultaneously maintaining a high vacuum in the column. A special detector is able to use gas ionization and amplification of the secondary electron signal from the sample. The gas ionization in the chamber also suppresses the changing of the samples and therefore coating is no longer necessary in this mode. Conventional scanning electron microscopy (CSEM) samples usually require fixation, drying and coating (Robinson et al., 1987; Dykstra, 1992), which would, in this instance, remove secretions and prevent observations of their release.

The aims of this work are to provide data about the localization and composition of the secreted material of trichome types of Styrian oil pumpkin and the changing of the secretory composition during the ontogenetical development of each trichome type, and to discuss the possible ecophysiological role of the investigated trichomes.

MATERIALS AND METHODS

To establish the experiments, seeds were grown on Perlite and transferred into growth chambers under defined climatic conditions with a photoperiod of 12 h (PAR 400–700 μmol m⁻² s⁻¹). Day and night temperatures were 22 °C and 18 °C, respectively, the relative humidity was 70 %. The investigated plants were 2 weeks old and the leaves were between 3 mm and 4 cm long. Whole leaves (3 mm) and portions of leaves, which had a dimension of 25 mm² were used. The leaf pieces were sampled from the middle of the leaves.

Microscopical investigations

ESEM. This is the scanning electron microscopy mode with low vacuum in the chamber. Through the use of a Peltier cooling stage the sample temperature was set to 5 °C. The dynamic investigations were carried out under continuous conditions, which were 6 torr with a relative humidity of 80 %. The samples were examined uncoated with a gaseous secondary electron detector (GSED) within a water vaporous environment. The relative humidity around the sample could be varied through manipulation of sample temperature and/or pressure within the chamber (Kolb and Stabentheiner, 2003).

CSEM. This is the conventional scanning electron microscope mode with high vacuum (approx. 10⁻⁴ Pa) conditions in the chamber. Samples were prepared by chemical fixation with 2.5 % glutardialdehyde, dehydration through an alcohol series, critical point drying using CO₂ as a drying agent, mounted on SEM stubs, and sputter coated with gold (Robinson et al., 1987).

All samples for CSEM and ESEM were mounted on aluminium stubs (diameter 1-2 cm) with double-sided conductive tape and investigated under different conditions with a Philips XL30 ESEM using an acceleration voltage of 20 kV.

Light microscopy (LM). The main classes of metabolites in secreted material of glandular trichomes were observed in fresh and fixed hand-sections, using the following different histochemical tests. Sudan black B was used to localize total lipids (Lison, 1960), Nile blue A was used for neutral and acidic lipids (Jensen, 1962), osmium tetroxide for unsaturated lipids (Jensen, 1962), neutral red for total lipids (Clark, 1981), Naturstoffreagenz A (β-aminodiethylester of diphenylboric acid) for detection of flavonoids (under UV 365 emission LP 397) (Wollenweber, 1982), FSA (fuchsin–safranin–astrablue) (fuchsin and safranin stain red for lignified and suberized cell walls and astrablue stains blue for non-cutinized cell walls), NADI (naphthol + dimethyl-paraphenylenediamine) reagent for lipids and terpenes (David and Carde, 1964), ruthenium red for pectins (Johansen, 1940).

The observations were made under a Zeiss microscope: Zeiss Axioskop equipped with a 100-W mercury arc lamp with colour video camera (Sony DXC 930 P with Sony control system) and a frame grabber (ITI MFG-3M-V with variable scan module AM-VS-and colour recording module AM-CLR-VP; Imaging Technology Inc.) was used to obtain digital images.

The images were obtained through Zeiss ×40 dry objective (n.a., 0.75), Zeiss ×63 dry objective (n.a., 0.95) and Zeiss ×100 oil immersion objective (n.a., 1.4), and the lengths of trichomes were measured and statistically analysed (image analysis system, Optimas 6.5.1; BioScanCorp.).

RESULTS

The adaxial and abaxial surfaces of the investigated leaves of Styrian oil pumpkin showed numerous glandular trichomes and bristle hairs. Each of the four trichome types of Cucurbita pepo var. styriaca could easily be characterized. They differed in size and structure, composition of the metabolites and the secretory process from the head and neck regions. Every type started its development with an epidermal cell, which first underwent a periclinal cell division.

Three of the four trichomes were capitate (I, II and IV), the last one characterized as ‘columnar digit’ trichome type (III). The samples as a whole, especially the glandular trichomes, showed a structural stability, and details of the cuticle were more visible than in the CSEM mode, due to the absence of water film. Only the bristle trichomes showed detriments.

Type I was the well-described short-stalked Cucurbita trichome (Fig. 1) with a length of 110 μm (±33) (mean value ± s.d.) and could be found on both leaf sides. This
Fig. 1. (A–E) ESEM micrographs and (F) CSEM micrograph showing the morphology and secretory products of glandular trichome type I on the leaves of Styrian oil pumpkin. (A) High density of different trichome types and the beginning of a secretory release; (B) contact of type I trichomes with bristle hairs (arrow) or with the same trichome type (arrowhead); (C) contact with a long-stalked type II (arrow); (D) secretory release in the apical region (arrow); (E) shrinkage in the apical region (arrow) and changing artefacts; (F) secretory stage in CSEM mode. (G–M) Bright field and fluorescence micrographs of the leaf trichomes of Styrian oil pumpkin showing histochemical characterization of secretory products: (G) the short-stalked trichome type in vivo; (H) three-celled stage, which stains blue for non-cutinization and red for lignification with the staining reagent FSA; (I) mature trichome, showing cutinization and lignification in the cell walls of the head and intermediate cell areas stained with FSA; (J) mature stage – the ‘middle cell’ is coloured red with the staining reagent Neutral red; (K) mature stages stained black-brown with osmium tetroxide; (L) staining for flavones with Naturstoffreagent A – secretory stage with an orange fluorescence and post-secretory stage only showing a coloration of the cell wall area (arrow head); (M) NADI staining for terpenes is positive in mature trichomes. Scale bars: A–F = 20 μm; G–J and L = 50 μm; K = 100 μm.
type consisted of a basal cell, a uniseriate stalk and a four-celled head region (Fig. 1G). The ESEM micrographs showed the secretory process of short-stalked glandular trichomes (Fig. 1A–D). This process started when the trichome was turgent (Fig. 1A). Then the mature trichomes had contact with bristle hairs (Fig. 1B, arrow and arrowhead), the epidermis or other trichomes (Fig. 1C, arrow). The secretory process depended on the density of trichomes and on the linking together of trichomes. Secretion was continuous during observation within ESEM and only involved apical cells in contact (Fig. 1D, arrow).

As a consequence of secreting, the trichome quickly dehydrated. It was observed that the structure collapsed, first only in the apical area, then step by step the other parts of the trichome lost their stability, followed by shrinkages and changing artefacts (Fig. 1E, arrow). Such secretory films could not be observed if type I was investigated under the CSEM mode as only a snapshot of pre- or post-secretory stages would be seen (Fig. 1F). In the CSEM mode the trichomes of type I did not show fixation or preparation damages.

Histochemical investigations with FSA (which stains red for lignified, suberized and cutinized cell walls and blue for uncutinized cell walls) featured different reactions pertaining to the differences in the ontogenetical development (Table 1, type I). From the one-cell stage to the four-celled stage the staining was faintly positive and no cutinization could be observed (Fig. 1H). After further divisions, which resulted in a four-celled head region (on a short-stalked and a basal cell) the cell walls showed added lignification. During head development, changes in cutinization could be observed. Young, but fully developed short-stalked trichomes featured a lignified head, but no cutinization of this region could be observed. After elongation, an adult trichome showed red staining indicating cutinization (Fig. 1I). Besides the head region the ‘intermediate cell’ was also lignified and cutinized. Histochemical investigations with neutral red that stains for total lipids were not conducted further, because of different behaviours in the colour intensity in the head region. However, in the ‘middle cell’ of a mature trichome clear pink coloration was visible (Fig. 1J). The head area of type I stored lipids, which could be identified by the staining reaction with osmium tetroxide. The intensity varied depending on the stage of development. Young trichomes with a lower cell number were faint-coloured, whereas mature ones were intensively black-brown (Fig. 1K). Staining with Sudan black B for lipids was slightly positive in all ontogenetical studies. Fully developed trichomes showed a weak reaction in the areas near the cell walls.

The secretory four-celled head showed up in the developing stage, becoming stained fluorescent orange with the Naturstoffreagent A (Fig. 1L) which indicated flavones. When the trichomes were fully developed, the compounds were volatilized and only residues were responsible for an orange fluorescence in the cell wall area (Fig. 1L, arrowhead). The NADI reaction for terpenoids showed up only in mature trichomes as an intensive blue-violet (Fig. 1M).

The long-stalked glandular trichome, the ‘neck-cell’ type (type II) (Fig. 2) could be found on both leaf sides and was more frequent on the leaf veins. This type was characterized by the two head cells, which were separated from the stalk region, normally containing five cells and one basal cell (Fig. 2G). The fully developed trichome had a length of about 345 μm (±41). Long-stalked, glandular trichomes were situated between bristle hairs and different kinds of glandular trichomes. Most of them showed the same developing stages (Fig. 2A). In the present investigations, young, underdeveloped trichomes were more stable than fully developed ones, because the cooling of the Peltier element was not efficient enough to cool the whole long-stalked trichome. They showed more inclination than other trichomes and this resulted in an unavoidable shrinkage when the secretory process started. When the ‘neck-cell’ types were fully developed (Fig. 2B, arrow) they leaned onto other trichomes at the top of the head and, due to this contact, secretory droplets could be observed during ESEM conditions (Fig. 2C, arrow). The apical areas showed a special structure that was detectable with the light microscope, too. These structures were not associated with the secretory process and they were more easily observed in CSEM samples than in ESEM probes, because a water film covered the head in ESEM. In Fig. 2D (arrow) a post-secretory stage was visible, where the apical cell collapsed inside after secretion. During the investigations in the CSEM mode, fully turgent trichomes with this special structure in the apical region (Fig. 2E) were found; however, the secretion process had already happened (Fig. 2F, arrow).

The composition and the localization of the secretory compounds differed depending on the stage of development (Table 1, type II). Until the mature stage of type II was reached, only a faint-coloured reaction for FSA staining could be detected. Although the pre-stages were lignified the density of lignification was weaker than that of mature trichomes and there was no reaction for cutinization. The mature trichomes showed a well-developed head region, which was cutinized and lignified. The stalk region was partly cutinized. The apical cell of the two-celled head area was more lignified compared with the upper cell. The ‘neck-cell’ area and the following upper cells were cutinized and lignified, whereas the basal cell was not cutinized (Fig. 2H). In contrast to the short-stalked trichome the reaction with Neutral red, staining for lipids, was clearly positive in mature trichomes. It depended on the developmental stage. The first dividing cell showed no reaction, the following stages had big vacuoles and the staining was aligned to these structures. The coloration was clearly positive in the apical cell, and a pink reaction was given in the ‘neck-cell’ area (Fig. 2I). Staining with osmium tetroxide (Fig. 2J) showed positive results with the same coloration stages as were described for Naturstoffreagent A. In younger stages, trichome type II only featured staining with Sudan black B in the lower cell of the head region (Fig. 2K). The fully developed trichome exhibited a reaction in the whole head area. The content of flavones in the head region was demonstrated with Naturstoffreagent A, showing a yellow-orange fluorescence (Fig. 2L). In younger long-stalked trichomes, flavones could be observed in the
TABLE 1. Histochemistry of the short-stalked trichome type I and the long-stalked, 'neck-cell' type II of Styrian oil pumpkin glandular trichome (two cells in the head and five cells in the stalk)

<table>
<thead>
<tr>
<th>Staining procedure</th>
<th>Target compounds</th>
<th>Observed colour</th>
<th>Staining results</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSA</td>
<td>Lignin</td>
<td>Red</td>
<td>+ + + + + +</td>
</tr>
<tr>
<td></td>
<td>Cutin</td>
<td>Red</td>
<td>+ + + + + +</td>
</tr>
<tr>
<td></td>
<td>Total lipids</td>
<td>Brownish</td>
<td>+ + + + + +</td>
</tr>
<tr>
<td></td>
<td>Unsaturated lipids</td>
<td>Dark blue to black</td>
<td>+ + + + + +</td>
</tr>
<tr>
<td></td>
<td>Sudan black B</td>
<td>Dark blue to black</td>
<td>+ + + + + +</td>
</tr>
<tr>
<td></td>
<td>Neutral red</td>
<td>Yellow</td>
<td>+ + + + + +</td>
</tr>
<tr>
<td></td>
<td>Osmium tetroxide</td>
<td>Blue</td>
<td>+ + + + + +</td>
</tr>
<tr>
<td></td>
<td>Neutral red</td>
<td>Yellow to orange</td>
<td>+ + + + + +</td>
</tr>
<tr>
<td></td>
<td>Terpenes</td>
<td>Violet-blue</td>
<td>+ + + + + +</td>
</tr>
</tbody>
</table>

Fully developed glandular trichome (two cells in the head and five cells in the stalk)

<table>
<thead>
<tr>
<th>Staining procedure</th>
<th>Target compounds</th>
<th>Observed colour</th>
<th>Staining results</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSA</td>
<td>Lignin</td>
<td>Red</td>
<td>+ + + + + +</td>
</tr>
<tr>
<td></td>
<td>Cutin</td>
<td>Red</td>
<td>+ + + + + +</td>
</tr>
<tr>
<td></td>
<td>Total lipids</td>
<td>Brownish</td>
<td>+ + + + + +</td>
</tr>
<tr>
<td></td>
<td>Unsaturated lipids</td>
<td>Dark blue to black</td>
<td>+ + + + + +</td>
</tr>
<tr>
<td></td>
<td>Sudan black B</td>
<td>Dark blue to black</td>
<td>+ + + + + +</td>
</tr>
<tr>
<td></td>
<td>Neutral red</td>
<td>Yellow</td>
<td>+ + + + + +</td>
</tr>
<tr>
<td></td>
<td>Osmium tetroxide</td>
<td>Blue</td>
<td>+ + + + + +</td>
</tr>
<tr>
<td></td>
<td>Neutral red</td>
<td>Yellow to orange</td>
<td>+ + + + + +</td>
</tr>
<tr>
<td></td>
<td>Terpenes</td>
<td>Violet-blue</td>
<td>+ + + + + +</td>
</tr>
</tbody>
</table>

(+) Finally positive, –, negative, +, positive.

'neck-cell' area and in the head area, too. When the trichome matured, the secretory process started and only the cell wall showed fluorescence.

The staining with NADI showed that secretion production starts in the 'neck-cell' area and investigations of different developmental stages showed the terpene content in the two head cells (Fig. 2M).

The ontogeny of the third trichome, type III, the 'columnar-digit' type (Fig. 3), started with a single epidermal cell, as well. This cell stayed as a 'columnar-cell' in the apical region of the trichome. The fully developed trichome had a length of 235 μm (± 49) and consisted of a two-celled head region, a stalk with three cells and one basal cell and occurred on both leaf sides (Fig. 3A). This 'columnar-digit' trichome showed no secretion during the ESEM observations and no inclination towards other trichomes was observed (Fig. 3B). In contrast to materials prepared for ESEM, individual cells of this non-glandular trichome were apparent in material prepared and fixed for CSEM (Fig. 3C, arrow).

The 'columnar-digit' trichomes showed differences in the staining results compared with the other trichome types (Table 2, type III). Staining with FSA showed up faintly in the first and second cell stages of lignification, but no sign of cutinization could be observed (Fig. 3D). Strongest lignification and cutinization was found in mature trichomes. Although staining with osmium tetroxide provided negative results, staining with Sudan black B showed a pale dark-blue coloration in cytoplasmatic areas near the cell walls during ontogeny. No flavones could be observed after staining with Naturstoffreagent A, whereas, the terpene reaction was faintly detectable in all ontogenetical stages.

Type IV, the 'stipitate-capitate' also started development with a single epidermal cell. Further, periclinal and anticlinal cell divisions resulted in a clear distinction between head area, stalk and base. While type IV, with a length of 1466 μm (± 253), was found on both leaf sides, it was primarily located on the abaxial leaf edges (Fig. 4). The base was an emergence, containing mesophyll cells, which were framed by the surrounding epidermal cells (Fig. 4G). After bringing the samples into the microscope chamber, secretion started immediately, and is demonstrated in Fig. 4A–E. Figure 4A shows the pre-secretory stage of the head area with its secretion centre (arrow). From above, a circle could be detected inside the glandular head and the secretion began in the head with a small droplet (Fig. 4B, arrow). During the investigation, the secretion area increased and a large droplet emerged very quickly (Fig. 4C, arrow head). Changing artefacts and shrinkage in the head could be observed after the secretion process, primarily in the epidermal structures surrounding the base (Fig. 4D, arrow). At the end of the secretion process the whole structure collapsed (Fig. 4E, arrow). The fixed material also showed that this trichome type possessed a central secretory area. With this material, however, it could not be determined at what stage of the secretory process the trichome was prior to sample preparation (Fig. 4F, arrow). It could be assumed that such big droplets, as they were observed in the ESEM mode, were washed off during preparation, as the secretion material was visible on fresh leaves before the material was
Fig. 2. (A–D) ESEM micrographs and (E and F) CSEM micrographs showing the morphology and the secretion release of glandular trichome type II on the leaves of Styrian oil pumpkin. (A) Different developing stages of type II. (B) Mature long-stalked trichome type with ‘neck-cell’ area (arrow). (C) Secretory droplets in the apical region (arrow). (D) Post-secretory stage after the secretion release (arrow). (E) Apical cell region with a special cuticle structure. (F) Apical region after a secretory release (arrow). (G–M) Bright field and fluorescence micrographs of the leaf trichomes of Styrian oil pumpkin, showing histochemical characterization of secretory products: (G) the long-stalked trichome type in vivo; (H) cutinization and lignification of the cell wall of the head and stalk areas, stained with FSA; (I) the lower head cell reacts positively with Neutral red; (J) positive staining reaction with osmium tetroxide in the head cells; (K) weak reaction for lipophilic compounds, packed in the lower head cells stained with Sudan black B; (L) yellow staining of secretion in the head and ‘neck-cell’ area with Naturstoffreagent A; (M) blue staining in the head and ‘neck-cell’ area with the staining for terpenes. Scale bars: A and L = 100 μm; B, G, I, J, K and M = 50 μm; C–F and H = 20 μm.
fixed. This dynamic process could be observed during light microscopy as well.

In a pre-stage of the ‘stipitate-capitate’ trichome, staining with FSA revealed no cutinization, but rather lignification of the cell walls. The trichome elongated and the apical region was completely cutinized, the stalk and the base only partially (Fig. 4H).

The secretion products of this glandular trichome type revealed positive results for lipophilic substances (Table 2, type IV) in all developmental stages after dyeing with Sudan black B (Fig. 4I) and osmium tetroxide. The way of secretion was presented by osmium tetroxide staining. In the pre-secretory stage the head area was completely coloured. After the secretion process started (Fig. 4J, arrow), the compounds were released and the staining area was reduced (Fig. 4K, arrow). In the pre stages, when the base and stalk cells were not fully developed, Naturstoffreagent A gave an orange-yellow fluorescence to flavones. The positive reaction could be observed in the head, which was partly inside the base (Fig. 4L, arrow). When the trichome was mature the flavones were transported out of the base and delivered through the head (Fig. 4M), whereas the staining for terpenes was visible only at the top of the head area in mature trichomes. The NADI reaction resulted in a blue-violet staining of the secretory products, which were only localized during the pre-stage in the apical region (Fig. 4N).

No visible reaction for staining with Nile blue A or ruthenium red showed up on any of the four trichome types.

**DISCUSSION**

As in plants of most Cucurbitaceae species, the leaf surfaces of Styrian oil pumpkins show three glandular trichomes, one non-glandular trichome type and one bristle hair type, all of which possess an independently characteristic morphology, ontogeny, histochemistry and secretion process. Three different capitulate trichome types were found on Styrian oil pumpkin: type I, a short-stalked trichome with four cells in the head region, also described on the blooms of *Cucurbita* by Uphof and Hummel (1962); type II, a long-stalked trichome type with two cells in the head region; and type IV, the biggest of the four trichome types, the so-called ‘stipitate-capitate’, were characteristic of Styrian oil pumpkin. Type III is non-glandular and is a ‘columnar-digit’ trichome with five cells in a line. These types were either found on one or both sides (Kolb and Müller, 2003).

Many plants, especially Cucurbitaceae species, are hairy and these trichomes have important tasks, e.g. to protect leaves from insects, to insulate during cold weather, protect from direct sunlight or to inhibit the growth of some fungi. For us, questions arose as to why the leaves of Styrian oil pumpkin have such a vast, varying number of trichomes and what their functions are. To answer these questions the histochemical properties of the trichomes in the present study were investigated and compared with trichomes of other species of similar morphology.

The ESEM mode made it possible to do the investigations under more or less natural conditions so it could be assumed that the trichomes showed their natural behaviour. The trichomes were stable during the investigations, which gave information about the adaption of these trichomes to special situations. For an efficient investigation of the secretion processes in the ESEM mode it was important that whole leaves were investigated and that the microscope chamber was cooled with the Peltier element, otherwise the structures could collapse. When the leaves were cut, the samples were not stable and a quick dehydration was the consequence. However, trichomes were under internal pressure through their own turgor, and since exudates were generally released through cuticular pores, it was possible that secretion during ESEM investigation was somewhat artificial, especially in connection with the speed of the secretion process.

Most of the glandular trichomes and the bristle hairs of *Cucurbita pepo* were very long and scanning electron microscopy induced dehydration and changing artefacts (Kolb and Stabentheiner, 2003). The best investigative conditions for *Cucurbita* leaves and their structures were to have the chamber pressure at 6 torr with a relative humidity of 80 % (see also Tai and Tang, 2001). These parameters made it possible to work under humid conditions without water droplets on the leaf surface.

In CSEM studies glandular trichomes were observed on fixed material without an active secretory release. ESEM observations revealed secretion stages, where secretory material was exuded in combination with an interaction between short-stalked trichomes and different structures. It was important for the trichomes to have contact with other structures. The collapse of these leaf trichomes
could be a consequence of loss of turgor through the release of an exudate.

Histochemical tests were useful to localize in situ the main chemical classes of metabolites, which were present in plant secretions. The histochemical results indicated that the secretion material contained terpenes, flavones and lipids and that the cell walls of the head, ‘neck-cell’ and the stalk showed the presence of lignification and cutinization.

Observations on short-stalked trichomes (type I) showed that the region between the head area and the stalk cells react differently. The so-called ‘middle-cell’ was a weak point and showed cutinization and lignification, whereas the upper stalk cells showed no cutinization or lignification. Young stages featured no cuticle or strong lignification, either. Fully developed trichomes possessed thick cuticle and lignified cell walls. Such staining behaviour of the cuticle has also been described for glandular trichomes of *Phillyrea latifolia* (Oleaceae) (Gravano et al., 1998), which was morphologically similar to type I of Styrian oil pumpkin. They were stained with phluoroglucinol for lignin, which had a positive reaction only in mature and senescent trichomes. The presence, structure and the composition of this Oleaceae trichome type could be associated with a high tolerance of salinity, which illustrated an adaption to extreme conditions. The trichome type I of Styrian oil pumpkin was similar in height and structure to this *Phillyrea latifolia* type; however, the *Cucurbita* plants investigated usually did not grow under extreme conditions.

The histochemical reactions of the oil pumpkin’s short-stalked trichome resembled those of *Calceolaria adscenden*s (Scrophulariaceae). While trichome type I of Styrian pumpkin showed only a faintly positive reaction with Sudan black B in all trichome regions, the above-mentioned Scrophulariaceae type’s lipophilic nature of the secretion has been demonstrated by an intensively positive reaction. In the head area of type I, as well as in the head of the similar trichome type of *Calceolaria adscenden*s, flavones could be detected, which made the head the centre of the glandular system (Sacchetti et al., 1999). The secretory release into the environment by *Calceolaria adscenden*s has been known to happen through the partial rupture of the cuticle caused by natural conditions such as temperature, humidity or predator contact. The presence of flavonoid metabolites has also been observed in glandular trichomes of *Phillyrea latifolia* (Tattini et al., 2000).

Only the mature stage of type I showed positive results for terpenes. A similarly positive staining reaction was noticed in the two-celled glandular trichomes of *Calceolaria adscenden*s (Sacchetti et al., 1999), which showed nearly the same ontogenetical developmental stages. In *Teucrium scorodonia* (Lamiaceae), two different types of glandular trichomes were observed. Type I, with four secretory head cells, had similarities to type I of Styrian oil pumpkin as the mature glandular trichome type I of *Teucrium scorodonia* showed a positive result for terpenes, too (Antunes and Sevinate-Pinto, 1991).

The capitate glandular trichome type of *Nepeta cataria* (Lamiaceae) showed similarities with type I of pumpkin plants in structure and function – also synthesized terpenes.
Fig. 4. (A–E) ESEM micrographs and (F) CSEM micrograph showing the morphology and the secretion of glandular trichome type IV ‘stipitate-capitate’ on the leaves of Styrian oil pumpkin: (A) Pre-secretory stage with the secretory centre (arrow); (B) beginning of the secretory release (arrow); (C) the secretory droplet is getting bigger (arrowhead); (D) changing artefacts (white areas in the head region) and shrinkage in the head area (arrow); (E) the collapse of the structure after secretion (arrow); (F) CSEM micrograph, stage of secretory process cannot be determined (arrow). (G–N) Bright field and fluorescence micrographs of the leaf trichomes of Styrian oil pumpkin showing histochemical characterization of secretory products: (G) type IV in vivo; (H) cell walls are lignified in the pre-stage, the stalk region is only partially cutinized; (I) positive dyeing with Sudan black B; (J) staining with osmium tetroxide in the apical head area, secretory process is visible; (K) staining with osmium tetroxide, the apical head area shows an advanced secretory process; (L) the part inside the basement (arrow) reacts positively for flavones; (M) mature trichome reacts positively for flavones in the head; (N) the apical region of the pre-stage reacts positive for terpenes. Scale bars: A = 20 μm; B, C, D, E, H, I and N = 50 μm; F, G, J and M = 100 μm, K and L = 200 μm.
(Kolalite, 1998). In plants these metabolites played a significant role in interactions between the plant and herbivorous or pathogenous organisms, respectively (Levin, 1973). The lignification and cutinization of mature trichome type I protected the hair from dehydration processes. The positive histochemical reaction and the stability of the secretory products, which did not evaporate during the investigation of SEM, was further evidence of the presence of terpenes.

The cutinization and the lignification in the head area were stronger in comparison with the stalk, and these differences were also clearly detectable in CSEM. The shrinkage in the apical cells may have occurred after the post-secretory stage as a sign of secretory release. The correlation between the stability of the glandular head of this trichome type of Styrian oil pumpkin and the stability in ESEM mode seemed to be an adaptation for special natural conditions as it was described of *Phillyrea latifolia* (Gravano et al., 1998). The intercalations of different compounds in type I were a protection against dehydration and offered a stability during the investigations, too.

Type II showed positive reactions after staining with FSA, osmium tetroxide, Sudan black B, Naturstoffreagent A and NADI reagent in the stalk and head regions. Cell wall lignifications in glandular trichomes were not typical and only certain trichomes synthesized lignin, e.g. the capitate type I of *Salvia aurea* (Lamiaceae). On the leaves of *Salvia aurea*, two different types of capitate glandular trichomes were found. Type I, with a monocellular stalk and a two-celled head, had structural similarities to type I of Styrian pumpkin and revealed an intense fluorescence of the lateral stalk walls and the ‘neck-cell’, as well as within the head, after staining procedures with berberine–aniline blue for suberin and lignin. The oil produced by these glandular trichome types was also thought to be responsible for protection against herbivores and pathogens. Both capitate trichomes of *Salvia aurea* showed a positive reaction for flavone production, too (Serrato-Valenti et al., 1997). These components of essential oils of various species of *Salvia* played a role in the inter-plant allelopathy due to their high phytotoxicity (Muller and Muller, 1964).

For *Salvia officinalis* (Lamiaceae) (Corsi and Bottega, 1999), five distinct types of glandular trichomes (one peltate and four capitate) with varying sites, secretory modes and secretions have been described. Type III of the capitate trichomes was also a long-stalked, capitate hair that showed a positive reaction for lipids and terpenes. In comparison with the ‘neck-cell’ trichome type of Styrian oil pumpkin, a cup shape could also be observed. The head area of *Cucurbita pepo* type II showed a black-brown staining for lipids and only a faintly positive reaction in the ‘neck-cell’ region with osmium tetroxide. Concerning the further staining of lipids in Styrian oil pumpkin type II with Sudan black B, only the areas near the cell wall were dyed, and a faintly positive reaction in the glandular head was shown. *Plectranthus ornatus* (Lamiaceae) featured five morphological types of glandular trichomes, peltate and capitate trichomes. The long-stalked trichomes showed a morphological similarity to type II of Styrian oil pumpkin and a high content of essential oils were observed as is characteristic for Lamiaceae. Further investigations showed a clear staining of secretion droplets and a black dyeing in the ‘neck-cell’ area. A discussion arose as to whether the secretory products of *Plectranthus* are involved in the chemical defence of plants, or if they act as floral rewards to pollinators (Ascensão et al., 1999). Essential oil of a lipophilic nature was described for a series of glandular trichomes, e.g. *Origanum dictamnus* (Labiateae) (Boabadilis and Tsikos, 1982), which could be identified with Sudan black B and osmium tetroxide. Until now the specific functions of these oil components are not known.

Also flavone secretory compounds were detected in trichome II of Styrian oil pumpkin and both capitate types of the above-described *Plectranthus* contain flavones.

Staining for terpenes showed a positive reaction in the ‘neck-cell’ area and in the glandular head of Styrian oil pumpkin of trichome II, whereas in *Plectranthus ornatus* terpenes were located in the subcuticular space. This had never been found in *Cucurbita pepo* because these trichomes had never had subcuticular structures.

This long-stalked trichome type of Styrian oil pumpkin seemed to be as stable as type I. During the investigations no dehydration or shrinkage was detectable, because of the lignification and cutinization of the stalk and the head region. Droplets on the apical cell of the head region indicated a secretion process, which was observed during the investigations with ESEM. After secretion, the apical cell collapsed and the stalk shrank. The secretory droplets were also detectable in the apical cell that confirmed perfectly to the histochemical investigations, giving an explanation of secretion production and way of secretion. On fixed material, secretory droplets were detectable with difficulty, however, the bigger part was washed off during the preparation steps, as also described on the glandular heads of trichomes of *Salvia aurea* on CSEM micrographs (Corsi and Bottega, 1999).

Also *Sicana odorifera* (Cucurbitaceae) (Krings et al., 2002) showed similar trichome types to the above-described type II of Styrian oil pumpkin, which were very touch sensitive and included a secretion-filled cell on the top of the apical cell. If these trichomes were touched they released a sticky exudate. It seemed that the ‘neck-cell’ type of Styrian oil pumpkin possessed the same ability of cementing arthropods to the leaf surface. The ‘neck-cell’ was the weakest point and reacted differently compared with the stalk and to the head. Furthermore, long-stalked trichomes, e.g. of *Salvia blegapropylla* (Lamiaceae) (Bisio et al., 1999) also exhibited the release of secretory products by passing droplets through the intact cuticle followed by the rupturing of the cuticle, as was observed in Styrian oil pumpkin.

In Styrian oil pumpkin, the ‘columnar-digit’ trichome type III, with five cells in line (and a basal cell), had no clear distinction between the apical cells and the following upper cells. This type could not be considered to be a capitate trichome. Histochemical observations on the fully developed trichome showed a positive reaction for FSA and a weak reaction for Sudan black B and NADI. A morphological similarity between this type and the ‘digitiform’ trichome types of *Plectranthus ornatus* (Ascensão et al., 1999) exists. The ‘digitiform’ trichome did not consist of a
glandular head or a subcuticular space in either plant. The cell walls of type III showed a positive staining for lignification, in contrast to the cell walls of the Lamiaceae type that reacted positively for both polysaccharide and lipids. Generally the ‘digitiform’ trichomes of Plectranthus ornatus showed a similar morphology to type III of Styrian oil pumpkin while also showing differences in histochemical reactions.

As described above for Salvia officinalis, four different capitate trichome types could be investigated, with type II of Salvia officinalis looking similar to the ‘columnar-digit’ (type III) of Styrian pumpkin. But this type II is considered to be a capitate hair, and tests for lipophilic compounds, essential oils and flavonoids were positive. Other tests used for lipophilic compounds of type III of Styrian oil pumpkin were negative. In the trichomes of type II of Salvia officinalis the hydrophilic component predominated. The capitate trichome of Salvia officinalis showed chemical and mechanical defenses against predators (Corsi and Bottega, 1999), whereas the function of the ‘columnar-digit’ trichome of Styrian pumpkin featured neither a clearly positive staining for lipids nor a positive reaction for essential oils, and so an involvement in the plant protection was not assured.

Terpenes were not clearly located in the ‘columnar-digit’ trichomes of Styrian oil pumpkin but other histochemical tests, e.g. in Plectranthus trichomes, had a clearly positive result, showing a violet drop of secretory material in the stalk cell (Ascensão et al., 1999).

Compared with the other trichome types of Styrian oil pumpkin the ‘columnar-digit’ trichomes did not show a secretory process during the ESEM investigations. The histochemical reactions were either barely positive, or negative. These facts confirmed the independent behaviour of the type III trichome from all other trichomes in Cucurbita pepo. The specific functions of the ‘digit’ trichome are not yet known.

The fourth described type of Styrian oil pumpkin, the ‘stipitate-capitate’ was the biggest trichome and was located on the upper-surface edge of the leaf. The staining reactions for PSA, Sudan black B, osmium tetroxide, Naturstoffreagent A and NADI were positive in all stages of differentiation between head and base.

The staining procedure for flavones of the ‘stipitate-capitate’ type of Styrian pumpkin in all developing stages exhibited a positive fluorescence signal in the head, stalk and base area. In pre-stages, the base structure with a yellow-orange fluorescent character could be observed and the head region showed the same colour. Neighbouring epidermal cells elevated these secretory cells inside the basement. Further elongations during development led to a strong fluorescence at the trichome apex, whereas the base did not show any fluorescence. These results showed similarities with glandular trichomes of Fagonia species (Zygophyllaceae) (Fahn and Simony, 1996). At maturity, this type showed a secretory cell, surrounded by epidermal cells, which were elevated above the leaf surface as a consequence of their division and elongation. The secretory material included lipophilic material, pectins and other polysaccharides. The exact characteristics of the components were not described for the glandular trichomes of Fagonia (Fahn and Simony, 1996).

Investigations on various stages of trichome development were made. The way secretory products are produced, detected with the fluorescence microscope, was the same as described for type IV of Styrian oil pumpkin. The presence of these trichomes on Fagonia leaves was thought to be an adaptive character in arid conditions, which could also be true for type IV of the Styrian oil pumpkin.

Also Cannabinoids (Cannabaceae) were abundant with capitate-stalked trichome types with a tier of secretory disc cells, where lipophilic material was produced, subtending a large non-cellular secretory cavity. At maturity these trichomes reached a height of 150–500 μm which was also found in the present studies for trichome type IV. The layer of trichomes makes Cannabis less palatable to many herbivores and omnivores (Fairbairn, 1972). The thick coating of trichomes, that was observed in type IV, is unpleasant for many insects. Further, the ‘stipitate-capitate’ trichome type of Styrian oil pumpkin may play an important role in plant protection, because of its size, structure and the method of secretory release (see Results).

In conclusion, it can be summarized that, on the leaf surface of Styrian oil pumpkin, five different trichome types, i.e. one bristle hair, three capitate glandular trichomes and one ‘digit’ non-glandular trichome, were observed. Each of these trichomes had its specific structure and function and demonstrated a different way of secretion processing and release.

All capitate types consisted of a basal, stalk and head region and all of these parts differed in the number of cells. Type I consisted of a basal cell, a short stalk and a four-celled head region. Type II, the so called ‘neck-cell’ type, was characterized by a long stalk region and a two-celled head region. The third investigated trichome type, type III, the ‘acuminate-digit’, consisted of a short stalk and a bicellular head region, which showed only a small constriction with no clear distinction between the head and the stalk areas. The fourth trichome, type IV, the so called ‘stipitate-capitate’, was the biggest trichome type and was visible without the microscope. This trichome type was characterized by a multicellular base, a uniseriate stalk and a multicellular head region.

However, the composition of the secretory material in all glandular trichome types was similar with the exception of the ‘digit’ hair. The amount of compounds differed and the stability of the trichomes differed within a small range. In contrast to the leaves of Nicotiana tabacum (Solanaceae), only two morphologically different glandular trichome types could be found, but both were acting in very different ways (Meyberg et al., 1991). Therefore, the question arose as to why and for what Cucurbita pepo leaves need all these different trichome types. Further clarification of the functions and ecophysiological roles of these epidermal appendages is required.

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**LITERATURE CITED**


