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Morphology, histochemistry, and ultrastructure of foliar mucilage-producing trichomes of *Harpagophytum procumbens* (Pedaliaceae)

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Abstract: The morphology, histochemistry, and ultrastructure of foliar mucilage-producing trichomes of *Harpagophytum procumbens* are investigated using a combination of light and electron microscopy. The leaves of *H. procumbens* bear short and long glandular trichomes comprising 1 or 2 basal epidermal cells, a stalk of 1–3 cells, a short neck cell, and a head of 2–5 cells. These trichomes are distributed on both leaf sides with a greater abundance on the abaxial side. Both types of trichomes secrete copious amounts of secretion as droplets onto the leaf surface, and the release occurs through micropores in the head cuticles. The secreted material is mainly constituted of mucilaginous polysaccharides, in addition to phenolic compounds and total lipids. The stalk surfaces of long trichomes are densely covered by numerous micropapillae helping the mucilage droplets to slide onto the leaf surface. The short and long trichomes have a similar ultrastructure: the secretory head cells are cytoplasmically dense due to the abundance of mitochondria, ribosomes, small vacuoles, plastids, Golgi bodies, and elements of endoplasmic reticulum. Golgi-derived vesicles are developed in the head cells, and each plastid contains large starch grains.

Key words: Medicinal plant, morphology, histochemistry, mucilage-producing trichomes, secretion mode, ultrastructure

1. Introduction

The family Pedaliaceae comprises approximately 17 genera and 80 species. Eight genera and 31 species are indigenous to the semiarid regions of southern Africa (Judd et al., 2006; Mabberley, 2008). *Harpagophytum procumbens* (Burch.) DC. ex Meissn. (devil's claw) is a medicinal desert plant indigenous to southern and eastern Africa. It has long been used as a folk remedy for the relief of arthritis, lumbago, and muscular pain (Chrubasik, 2004) and has analgesic, antirheumatic, antiinflammatory, and hypoglycemic properties (Blumenthal et al., 2000; Mahomed and Ojewole, 2004).

There are a wide variety of secretory structures in plants, and these differ greatly with regard to their location on plant organs as well as the composition of the secretory product (Fahn, 1988). Studies have been conducted on the mucilage idioblasts in mesophytic and xerophytic species (Mollenhauer, 1967; Bouchet and Deysson, 1971; Trachtenberg and Fahn, 1981). The family Pedaliaceae is characterized by capitate, short-stalked, mucilage trichomes, which are very widely distributed and probably occur in all species (Solereder, 1908; Metcalfe and Chalk, 1972). Naidoo et al. (2012) found 2 morphologically distinct glandular trichomes on *Ceratotheca triloba* leaves. The first type is a long trichome with 8–12 basal cells of pedestal, 3–14 stalk cells, a neck cell, and a head of 4 cells in 1 layer. The second type is a short trichome comprising 1 or 2 basal epidermal cells, a uni- or bicellular stalk, and a head of 2–8 cells (Naidoo et al., 2012).

To the best of our knowledge, literature data on the leaf glandular trichomes of *H. procumbens* are lacking. Therefore, this study represents the first detailed report on the morphology, the histochemistry, and the ultrastructure of the foliar mucilage-producing trichomes of *H. procumbens* using microscopic techniques.

2. Materials and methods

2.1. Plant collection

Harpagophytum procumbens was collected from Kuruman in the Karoo, South Africa, and was cultivated in the greenhouse of the School of Life Sciences, University of KwaZulu-Natal (27°28′S, 23°28′E). A voucher specimen (Naidoo et al. *sn.*) was deposited in the Ward Herbarium of the University of KwaZulu-Natal. Leaves from 3 developmental stages, young, mature, and old, were used for this study. Ten replicates were investigated from different plants in each case.

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2.2. Electron microscopy

To investigate trichome morphology and distribution, leaf samples were observed by scanning electron microscopy (SEM). Leaf samples were freeze-dried in an Edwards Modulyo freeze-dryer at –60 °C at a vacuum of 10^{-2} Torr for 5 days. The samples were secured to brass stubs with carbon conductive tape, sputtered with gold, and observed under a JEOL JSM-6100 scanning electron microscope at 12 kV.

To validate the appearance of freeze-dried samples, fresh leaves were observed by environmental scanning electron microscopy with a Philips XL 30 environmental scanning electron microscope at 12 kV operating at low vacuum mode.

To view trichomes by transmission electron microscopy, leaf segments (1–2 mm^2) were prefixed overnight in Todd's fixative as the primary fixative, which contains 1.5% paraformaldehyde, 2.5% glutaraldehyde, 0.3% calcium chloride, and 0.03% picric acid in 0.1 M sodium cacodylate buffer at pH 7.3 (Todd, 1986). The tissues were washed with phosphate buffer and postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. Following ethanol dehydration, the samples were progressively infiltrated and then embedded in 100% low-viscosity resin (Spurr, 1969) and polymerized for 8 h at 70 °C. Ultrathin sections of 80 nm were picked up on uncoated 200 square mesh copper grids and poststained with 4% aqueous uranyl acetate and lead citrate (Reynolds, 1963). These sections were viewed with a JEOL JEM-1010 transmission electron microscope at 60 kV.

2.3. Light microscopy

For light microscopy, semithin sections (0.5–2.0 µm) of leaf samples provided by the transmission electron microscopy method were cut with glass knives using a Reichert Jung Ultracut-E Ultramicrotome. The sections were poststained with 0.5% toluidine blue-O dissolved in 0.1% sodium carbonate at pH 11.1 (Feder and O'Brien, 1968). Stained sections were mounted onto slides in LR White, viewed, and photographed with a Leitz light microscope.

2.4. Histochemistry

For the histochemical investigation, leaf segments (approximately 3 mm^2) were initially placed in formaldehyde (70%) for 48 h. Following ethanol dehydration, these samples were then placed in a graded series of xylene:ethanol mixtures for 1 h each before being placed in 2 xylene incubations for 15 min each. Paraplast Plus wax pellets (McCormick Scientific) were then added, with a gradual replacement of xylene by wax, in an embedding oven at 56 °C. The wax was left for 3 h at a time for 12 h in total. A final addition of wax was added for 3 h, readying blocks for sectioning. Wax sections were dewaxed by exposure to three 2-min baths of xylene (100%), three 2-min baths of ethanol (100%), a single 2-min bath in 70% ethanol, and a final 2-min wash in tap water. The dewaxed sections were then subjected to the histochemical stains.

Histochemical analyses were performed on dewaxed sections and hand-cut fresh leaves to detect the presence of mucilaginous polysaccharides, lipids, terpenes, and phenolics. To do this, the leaf segments were stained with: a) Ruthenium Red for mucilage (Johansen, 1940; Jensen, 1962; Bornman et al., 1969); b) Sudan III and IV (Sudan Red) and Sudan Black B for lipids (Lison, 1960; Pearse, 1985; Brudrett et al., 1991); c) NADI reagent and antimony trichloride for terpenoids (David and Carde, 1964; Hardman and Sofowara, 1972; Mace et al., 1974); and d) ferric trichloride and potassium dichromate for phenolics (Johansen, 1940; Gabe, 1968). For all the histochemical dyes used, control tests were carried out simultaneously according to the respective authors. These sections were examined and photographed using a Leitz light microscope (School of Life Sciences, University of KwaZulu-Natal).

3. Results

3.1. Morphology and distribution

The leaves of *Harpagophytum procumbens* are densely covered by glandular trichomes. Two types of short and long trichomes are found on both sides of mature leaves. These trichomes consist of 1–2 bulbous, basal epidermal cells; a stalk of 1–3 cells; a very short neck cell; and a multicellular head of 2–5 cells arranged in 1 layer. Short trichomes are either subsessile or present a very short unicellular stalk with a smooth surface (Figures 1A–1C). The glandular heads may consist of a variable number of cells: 2 cells divided by only 1 vertical wall (Figure 1A); 1 elongated cell and 2 triangular cells (Figure 1B); or 4 (rarely 5) triangular cells arranged like a cross with smooth surface, and walls projecting convexly at the margin of the head (Figure 1C).

The long trichomes have a short bicellular-to-long multicellular stalk with a micropapillate surface (Figure 1D–F). This stalk is composed of elongated cells at the base graduating into shorter cells above. The glandular heads may consist of 2 (Figure 1D), 3 (Figure 1E), or 4 (rarely 5) cells (Figure 1F).

Anatomical structure of the short and long trichomes is shown in Figure 2A–C. Figure 2A and 2B show the structure of short and long trichomes with 4-celled heads in longitudinal sections. Figure 2C shows the longitudinal section of the stalk and neck cells of long trichomes. In long trichomes, the stalk surface is densely covered by numerous micropapillae (Figure 2D).

Short trichomes with 4-celled heads occur in large numbers all over the leaf lamina, further exceeding the density of long trichomes (Figure 2E), present only along the leaf veins and margins. The glandular trichomes are

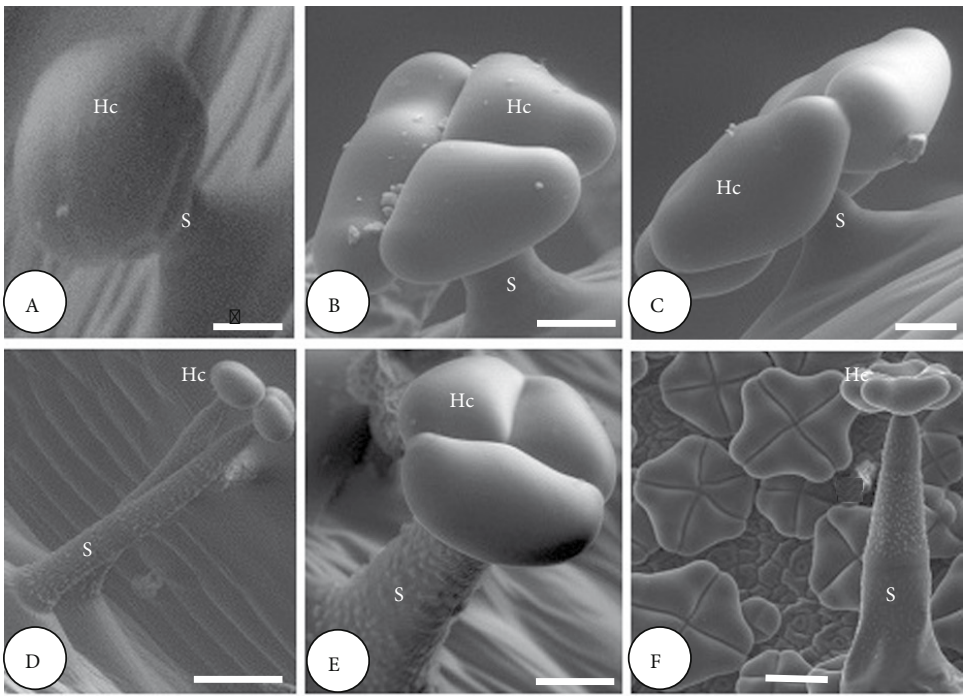

Figure 1. Electron micrographs showing morphology of glandular trichomes on *Harpagophytum procumbens* leaves. **A**- Short trichome with head of 2 cells; **B**- short trichome with head of 3 cells; **C**- short trichome with head of 4 cells; **D**- long trichome with head of 2 cells; **E**- long trichome with head of 3 cells; **F**- long trichome with head of 4 cells and top view of short trichomes with heads of 4 cells (rarely 5) visible in background. Scale bars: A–C = 10 µm; D = 50 µm; E and F = 25 µm. Abbreviations: Hc = head cell; S = stalk.

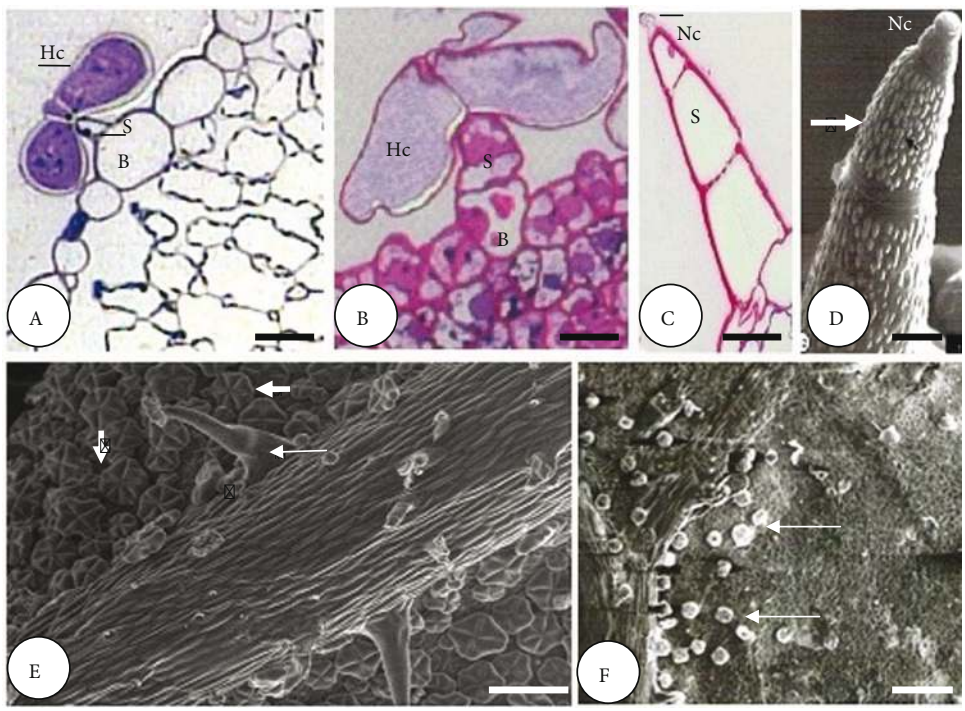

Figure 2. Anatomical structure and distribution of glandular trichomes on *Harpagophytum procumbens* leaf. **A, B**- Light microscopy micrographs of longitudinal sections showing structure of short and long trichomes with heads of 4 cells; **C**- light microscopy micrograph of longitudinal section showing structure of stalk with 3 cells in long trichome; **D**- electron micrograph showing numerous micropapillae on the stalk surface in long trichome (arrow) and neck cell; **E**- electron micrograph showing distribution of short trichomes (arrowheads) and long trichomes (arrow) with heads of 4 cells on abaxial surface of young leaf; **F**- electron micrograph showing distribution of glandular trichomes (arrow) on adaxial leaf surface. Scale bars: A–D = 10 µm; E = 200 µm; F = 250 µm. Abbreviations: B = basal epidermal cell; Hc = head cell; Nc = neck cell; S = stalk.

more abundant on the abaxial leaf surface (Figure 2E) than on the adaxial surface (Figure 2F). Young leaves are densely covered with trichomes that decrease as the leaf matures.

3.2. Secretion mode

The short and long trichomes display similar secretory behavior. They secrete copious amounts of secretion onto the leaf surface, mainly as droplets of variable size (Figure 3A–E); the secretion release occurs through micropores in the cuticle covering the head cells (Figure 3E–I). The micropapillae observed on the stalk surface of the long trichomes may help the secretory droplets to slide onto the leaf epidermis (Figure 3D). Following the secretion release, shrinkage of the head cells is generally observed in mature leaves (Figure 3F–H), and then the glandular cells degenerate (Figure 3I).

3.3. Histochemistry

In both short and long trichomes, histochemical testing with Ruthenium Red gave a strong positive response,

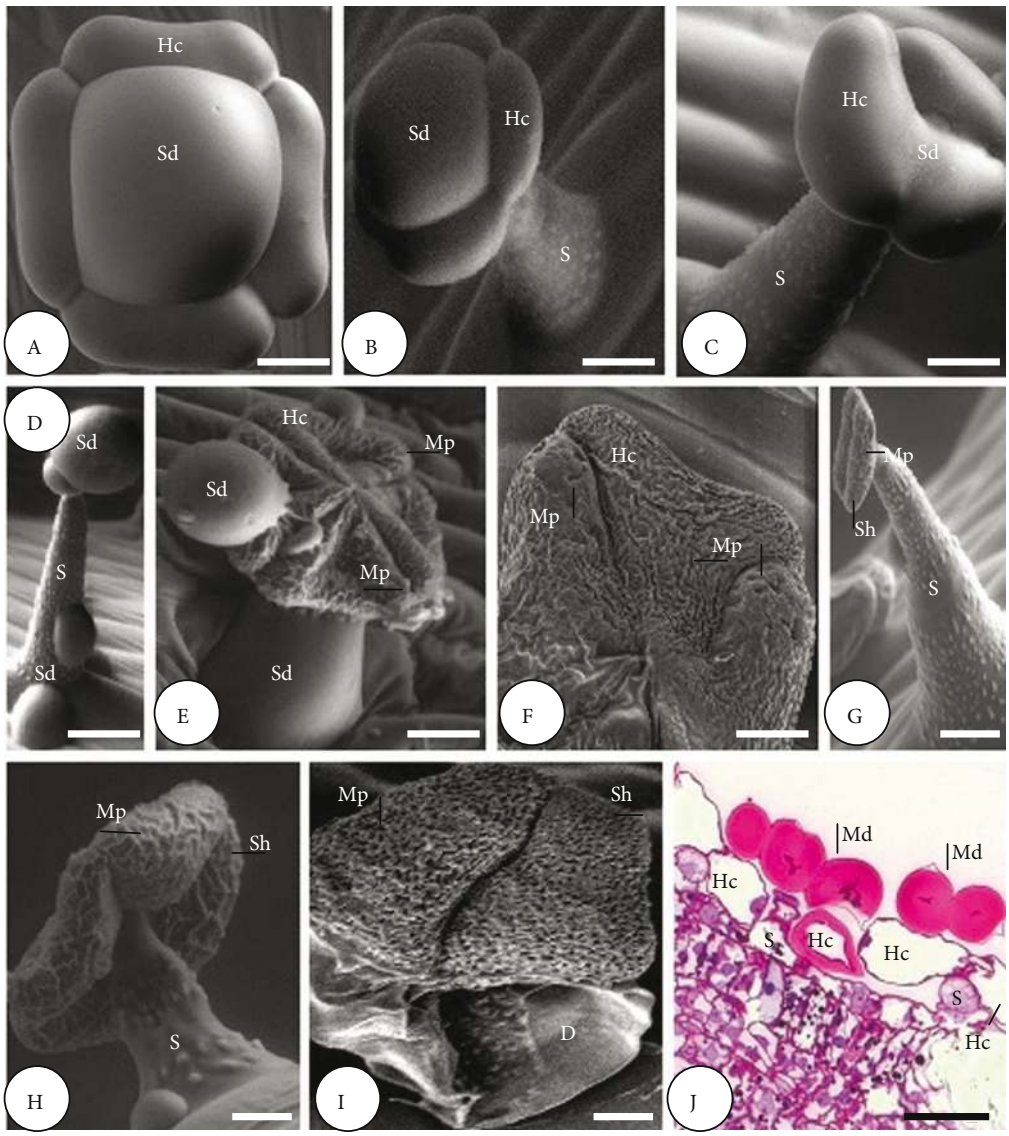

Figure 3. Electron micrographs showing secretion mode of glandular trichomes of *Harpagophytum procumbens*. **A, B, C**- Secretions cover head cells of short trichome with head of 4 cells and long trichomes with head of 4 cells and head of 3 cells, respectively; **D**- long trichome with secretion droplets sliding down onto the leaf surface; **E**- copious amounts of secretion secreted onto the leaf surface by short trichome with head of 4 cells through micropores in head cuticle; **F, G, H**- shrunken head cells after releasing secretions through micropores in head cuticles of short trichome with heads of 4 cells and long trichomes with head of 2 cells and head of 4 cells, respectively; **I**- head of short trichome with 2 shrunken cells and 2 degenerative cells; **J**- light microscopy micrograph of longitudinal section in long trichomes with heads of 4 cells showing intense pink coloration of secreted mucilage droplets and head cells with Ruthenium Red testing. Scale bars: A, C, I = 10 µm; B = 12.5 µm; D = 25 µm; E, H = 5 µm; F = 2.5 µm; G = 25 µm; J = 10 µm. Abbreviations: D = degenerative head cell; Hc = head cell; Md = mucilage droplet; Mp = micropores; S = stalk; Sd = secretion droplet; Sh = shrunken head cell.

showing intense pink coloration of the head cell cytoplasm and the secreted droplets on the leaf surface; these results clearly indicate the presence of mucilaginous polysaccharides (Figure 3J). Total lipids stained orange to red with Sudan Red or black with Sudan Black B. Phenolic compounds and tannins were evidenced by a brown color when stained with ferric trichloride and potassium dichromate, respectively. The 2 histochemical tests for terpenoids gave negative results. The test controls gave negative responses.

3.4. Ultrastructure

Ultrastructural studies reveal that the basal and stalk cells are highly vacuolated, with the cytoplasm displaced to the cell periphery (Figure 4A–C). The lateral walls of the stalk

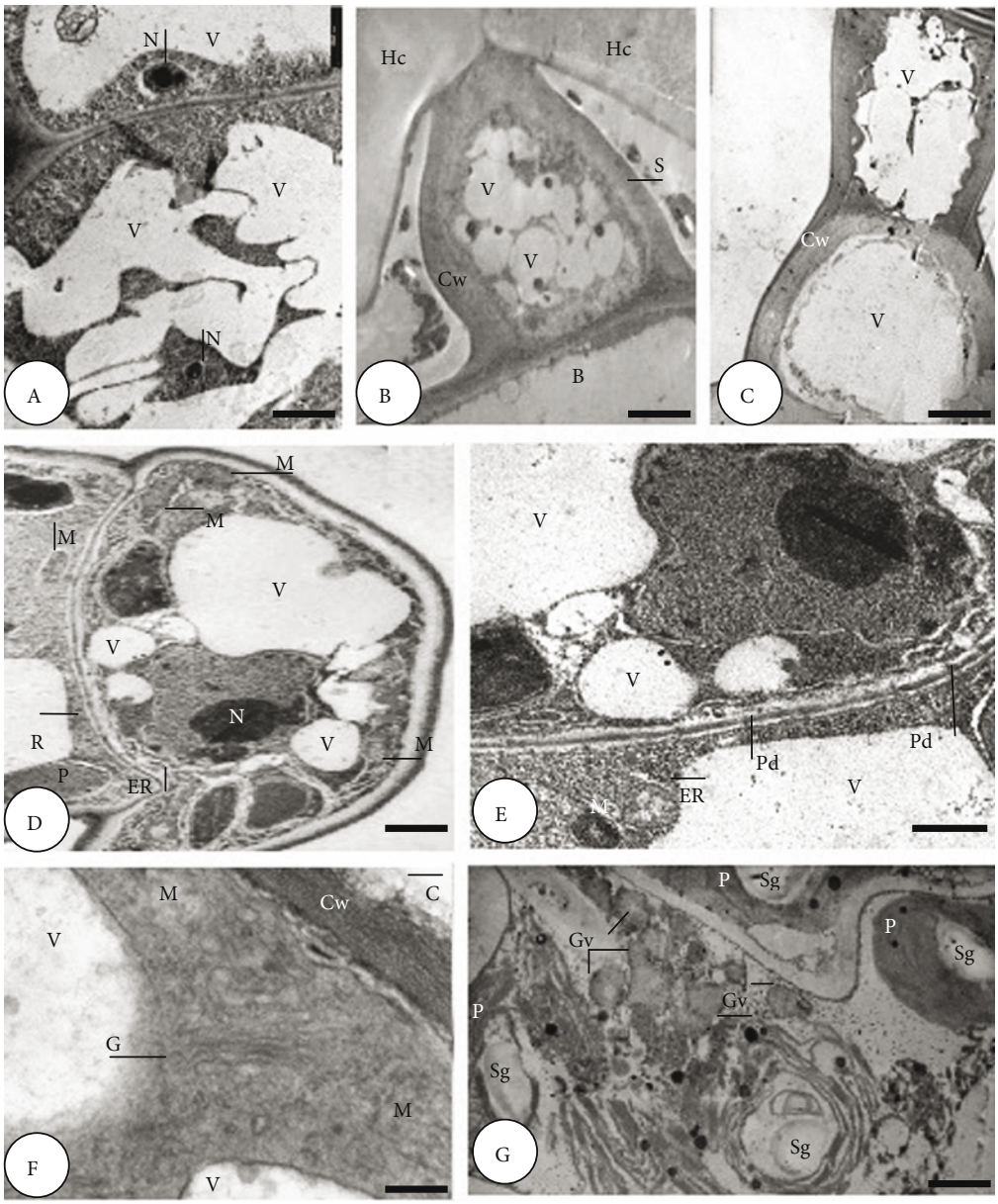

Figure 4. Electron micrographs of ultrastructure of glandular trichomes of *Harpagophytum procumbens*. **A**- Basal epidermal cell appears highly vacuolated with the cytoplasm displaced to the cell periphery; **B**- stalk cell of short trichome (with head of 4 cells) appears highly vacuolated with heavily cutinized lateral walls; **C**- 2 stalk cells of long trichome with head of 4 cells appear to be heavily cutinized; **D**- details of head cells of short trichome with head of 4 cells at presecretory stage; **E**- connecting cell walls of head cells showing numerous plasmodesmata; **F**- head cell showing Golgi body and mitochondria possessing a dense matrix and well-developed cristae, cell wall, and a head cuticle; **G**- head cell at secretory stage showing Golgi vesicles and plastids with large starch grains. Scale bars: A, G = 0.5 µm; B–D = 1 µm; E = 0.3 µm; F = 0.1 µm. Abbreviations: B = basal epidermal cell; C = head cuticle; Cw = cell wall; G = Golgi body; Gv = Golgi vesicles; ER = endoplasmic reticulum; Hc = head cell; M = mitochondrion; N = nucleus; P = plastid; Pd = plasmodesmata; S = stalk; Sg = starch grain; V = vacuole.

appear heavily cutinized (Figure 4B and 4C). The head cells possess dense cytoplasm due to the presence of prominent nuclei, abundant mitochondria and ribosomes, numerous small vacuoles, a high frequency of plastids, and elements of endoplasmic reticulum (Figure 4D and 4E). The head cells do not present a subcuticular space (Figure 4D). The adjacent walls of the head cells are thin (Figure 4D and 4E) and crossed by numerous plasmodesmata (Figure 4E).

Ultrastructural observations of the head cell cytoplasm showed Golgi bodies and mitochondria with a dense matrix and well-developed cristae near the cell wall (Figure 4F). At the secretory stage, numerous Golgi vesicles with a slightly electron-opaque material develop in the head cells, and each plastid contains a large starch grain that may be hydrolyzed to sugar (Figure 4G).

4. Discussion

The leaf surface of *Harpagophytum procumbens* is covered by short and long glandular trichomes, distinguished on the basis of cell number and morphology of the stalk. The glandular head is multicellular and composed of 2–5 cells. Solereder (1908) and Metcalfe and Chalk (1972) found mucilage-producing trichomes with heads of 4 or more cells in the family Pedaliaceae. Naidoo et al. (2012) observed a multicellular head of 2–8 cells in the short mucilage-producing trichomes of *Ceratotheca triloba* leaves. The surface of the stalk is smooth in the short trichomes, while it is densely covered by numerous micropapillae in the long trichomes. According to Werker (2000), trichomes may be smooth or exhibit microornamentation; these micropapillae help the mucilage droplets to slide onto the leaf surface. Roughness is used as the basis of an antiadhesive or water-repellent surface (Barthlott and Neinhuis, 1997; Neinhuis and Barthlott, 1997).

The present study revealed that the density of mucilage-producing trichomes was higher on the leaf abaxial surface than on the adaxial surface, which was characterized by scattered trichomes. Masrahi et al. (2012) also noticed that the mucilage glands are denser on the abaxial surfaces of *Sesamum alatum* leaves. The short trichomes with 4-celled heads were present all over the leaf lamina, exhibiting a higher density with respect to the long trichomes in the studied species. A similar distribution pattern was reported in *Ceratotheca triloba* for short glandular trichomes, which are predominant on the abaxial surface (Naidoo et al., 2012). Results also showed that trichome densities decrease as leaf age increases. Similar observations were made for *Ceratotheca triloba* and *Trichosanthes cucumerina* (Adebooye et al., 2012; Naidoo et al., 2012).

In the examined species, the secretion of both trichome types is released through micropores in head cuticles, mainly due to the pressure exerted by the copious amounts of secretion in the head cells. In addition, in the short glandular trichomes of *Ceratotheca triloba* the secretion release occurred via a porous head cuticle (Naidoo et al., 2012), whereas in the long trichome the secreted material is released through micropores located in a marked circular area on the upper part of each head cell.

In this study, very short neck cells were observed in both types of trichomes. In several Lamiaceae species, the lateral walls of the neck cells of capitate trichomes are equipped with suberin-like substances, which likely prevent the back-flow of secreted products into the mesophyll tissue (Fahn, 1988; Serrato-Valenti et al., 1997; Ascensão et al., 1999). The ultrastructural studies on *H. procumbens* trichomes also show that the lateral walls of the stalk cells are heavily cutinized. These observations are consistent with previous reports on the glandular trichomes of several xeromorphic species characterized by the cutinized lateral walls of the stalk cells (Fahn, 1988; Ascensão and Pais, 1998; Werker, 2000).

Histochemical tests are useful tools to detect and localize the main chemical classes of metabolites present in plant secretions. The histochemical results indicated that the secretion of both short and long trichomes of *H. procumbens* contained mainly mucilaginous polysaccharides, in addition to phenolic compounds and total lipids. This is consistent with the results of Barone et al. (1996), who found that the mucilage secretion of *Dicerocaryum zanguebaricum* (Pedaliaceae) was mainly constituted of homogeneous polysaccharides. Naidoo et al. (2012) proved that short trichomes produced larger amounts of mucilaginous polysaccharides, whereas long trichome secretion was mainly characterized by unsaturated lipids.

The exact role of these substances is not clear; however, the presence of copious amounts of viscous, adhesive polysaccharides suggested that the secretions may act as lubricants to facilitate leaf expansion (Modenesi et al., 1984) or may be involved in the chemical defense of trapping insects by stickiness (Gregory et al., 1986; Sutherst and Wilson, 1986; Corsi and Bottega, 1999; Werker, 2000). The viscous secretions resulting from the interaction of polysaccharides with phenolic compounds trap phytophagous insects, encasing the legs and tarsi (Gibson, 1971; Tingey and Gibson, 1978; Naidoo et al., 2012). The shiny appearance of mucilaginous secretions may serve in light reflection, reduction of leaf temperature, and water loss by transpiration (Dell and McComb, 1978; Fahn, 1979; Werker and Fahn, 1981; Gaff, 1997; Ascensão et al., 1999; Hachfeld, 2003; Wagner et al., 2004).

The presence of prominent nuclei and a highly organized cytoplasm with abundant mitochondria, plastids, Golgi bodies, endoplasmic reticulum, and numerous small and variously shaped vacuoles in the head

cells of *H. procumbens* mucilage-producing trichomes are typical ultrastructural features of secretory tissues with high metabolic activity (Karabourniotis and Fasseas, 1996; Turner et al., 2000). There was a high frequency of plastids with large starch grains that may be hydrolyzed to sugar at the secretion stage, as occurs in other secretory structures, such as the nectariferous cells of several plant species (Durkee et al., 1981; Zer and Fahn, 1992; Fahn and Shimony, 2001).

Ultrastructural observations of the short and long trichomes showed the presence of abundant Golgi vesicles in the head cells at the secretory stage. Discharge of these vesicles may occur by the granulocrine pathway (Fahn, 1979, 1988; Ascensão et al., 1999; Beck, 2010), where the membrane of a vesicle fuses with the plasmalemma, releasing its contents to the external surfaces. Trachtenberg and Fahn (1981) confirmed that only Golgi bodies were involved in mucilage secretion and also suggested that the mucilage passed through the plasmalemma by the granulocrine pathway. A similar secretion mode was also proven in the short mucilage-producing trichomes of *Ceratotheca triloba* (Pedaliaceae) (Naidoo et al., 2012), whereas in *Ceratotheca sesamoides* the secretion was released following the lysis (Abels, 1975) or rupture (Ihlenfeldt, 2001) of the outer walls of the head cells. The glandular cells also function as temporary storage sites of the secreted material without the formation of subcuticular spaces, as observed in the typical glandular trichomes of Lamiaceae (Serrato-Valenti et al., 1997; Ascensão and Pais, 1998; Ascensão et al., 1999).

The morphological characteristics of trichomes observed on leaves of *Harpagophytum procumbens*, compared with trichomes on *Ceratotheca triloba* leaves, can be used in plant systematic studies in Pedaliaceae.

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