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The Structure of Colleters in Several Species of *Simira* (Rubiaceae)

DENISE ESPELLET KLEIN[1], VALDIRENE MOREIRA GOMES[2], SEBASTIÃO JOSÉ DA SILVA-NETO[3] and MAURA DA CUNHA[1,*]

[1]*Laboratório de Biologia Celular e Tecidual, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense,* [2]*Laboratório de Bioquímica e Fisiologia de Microrganismos, CBB, UENF and* [3]*Fundação Margareth Mee/ Programa Mata Atlântica, Instituto de Pesquisas Jardim Botânico do Rio de Janeiro, Brazil*

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• *Background and Aims* Colleters are secretory structures consisting of a parenchymatic middle axis surrounded by a layer of palisade-like epidermal cells. Colleters occur in a large number of rubiaceous species. Their function is to protect the developing shoot apex. They are also taxonomically useful in the Rubiaceae. This study characterized the structure of the colleters of *Simira glaziovii*, *S. pikia* and *S. rubra* and the biochemistry of secretions in *S. glaziovii*.
• *Methods* Stipules of the shoot apices of the three species studied were collected at Barragem de Saracuruna, in Rio de Janeiro state, Brazil. The samples were fixed according to the usual methods for light and electron microscopy. Secretion stipules of *S. glaziovii* were washed with 0·1 M Tris–HCl plus 0·1 %Triton X-100 to extract proteins and carbohydrates.
• *Key Results* Colleters in these species are located at the base of the stipule. Each species shows a different pattern of distribution. They form as emergentia from the stipules. *Simira glaziovii* was different from the other two species because it exhibited vascular traces. The epidermal cells of colleters have dense cytoplasm, nuclei, small vacuoles, endoplasmic reticulum, Golgi apparatus, mitochondria and extraplasmic spaces if they are secretory. The outer cell wall of the mature colleters differs from the outer cell wall of stipule cells and immature colleters. Both carbohydrates and proteins were found in secretions from the stipules of *S. glaziovii*.
• *Conclusions* Few ultrastructural differences were noted among the three species. These secretory structures not only protect the shoot apex, but also have taxonomic importance below the genus level.

Key words: Colleters, secretory structure, microscopy, plant anatomy, ultrastructure, development, biochemistry, *Simira*, Rubiaceae.

INTRODUCTION

The colleter, an epidermal secretory structure, can be found mainly on the adaxial side of stipules and/or sepals among 60 families of the angiosperms (Thomas, 1991). These structures have been regarded as trichomes (Horner and Lersten, 1968), but develop from both protoderm and other ground elements as emergentia. Other names have been reported for these structures including 'squamallae' (Ramayya and Bahadur, 1968) and 'stipular glands' (Van Hove and Kagoyre, 1974).

Most of the functional aspects of these secretory structures are unknown. Several authors report that colleter secretions cover and protect the developing shoot apex (Williams *et al*., 1982; Thomas and Dave, 1989, 1990). In nodulated rubiaceous species, however, the secretions of colleters are surmised to play a vital role in the nutrition of symbiotic bacteria (Van Hove and Kagoyre, 1974; Lersten, 1975). They might also act as a pathway for bacterial entry into the leaves (Miller *et al*., 1983). In contrast, some secretory structures have evolved as defense against pathogens and insects (Farrell *et al*., 1991; Zalucki *et al*., 2001; Cruz *et al*., 2002). Insects and pathogens that attack secretion-producing plants are faced with a combination of both physical barriers and chemical defenses provided by exudate-secreting structures (Giordani and Lafon, 1993; Wititsuwannakul *et al*., 2002; Azarkan *et al*., 2003).

The anatomical structure of colleters can be described as a parenchymatic cellular axis surrounded by a layer of palisade-like epidermal cells (Thomas, 1991; Da Cunha and Vieira, 1997). The epidermal cells of colleters are known to be secretory. Occasionally, the middle axis develops vascular bundles (Thomas, 1991). Horner and Lersten (1968) and Lersten (1974*a*, *b*) described four types colleters in the Rubiaceae based on the appearance of their epidermis: standard, reduced, brush-like and dendroid. The standard type is composed of compact epidermal cells. It is the most common type and shows some variability among species in size and in constrictions at their bases (Robbrecht, 1988).

Colleters are located on the adaxial surfaces or margins of stipules in a large number of species of the Rubiaceae. These secretory structures not only protect the shoot apex, but also have taxonomic importance below the familial level (Robbrecht, 1988). Rubiaceous species are one of the most important components of the plant community of the Atlantic Rain Forest at Macaé de Cima in the state of Rio de Janeiro (Lima and Guedes-Bruni, 1997). The family is also important ecologically (Guedes-Bruni, 1998).

This study was performed to further the understanding of the anatomical structure and secretions of the colleters of *Simira glaziovii*, *S. pikia* and *S. rubra*.

* For correspondence. E-mail maura@uenf.br

MATERIALS AND METHODS

Botanical material

Stipules of the shoot apices of *Simira glaziovii*, *S. pikia* (K. Schum.) Steyerm and *S. rubra* (Mart.) Steyerm were collected in Barragem de Saracuruna, in the city of Duque de Caxias, in Rio de Janeiro state, Brazil, during the months of March, April and December in 2001. Stipules of *Simira glaziovii* were used for studies of development and secretory composition. For comparison with *Simira glaziovii,* only one stage of development of colleters in *S. pikia* and *S. rubra* was observed by microscopy.

Light microscopy

Whole stipules or stipular fragments were fixed for 2 h in a solution of 2·5 % glutaraldehyde and 4·0 % paraformaldehyde buffered with 0·05 M cacodylate buffer to pH 7·2. Subsequently, the samples were rinsed three times with buffer and post-fixed for 2 h at room temperature with 1·0 % osmium tetroxide in 0·05 M cacodylate buffer to pH 7·2. The post-fixed samples were dehydrated in a graded series of acetone solutions (30, 50, 70, 90 and 100 %; 1 h each). The material was infiltrated and embedded in the epoxy resin Epon (Polybed). Microtome sections (1·0 μm) were cut and stained with toluidine blue (0·05 % aqueous solution). The slides were sealed with Entellan® (Merk) and examined with an Axioplan ZEISS microscope.

Transmission electron microscopy

The stipule fragments were fixed, post-fixed, dehydrated and embedded as described above. Ultrathin sections were collected with 300-mesh grids, stained with 1·0 % uranyl acetate followed by 5·0 % lead citrate for routine observation. Three other stains were used to elucidate the cytochemistry of colleter cells: (1) imidazole-buffered osmium tetroxide to enhance and observe the preservation and contrast of lipids (Angermüller and Fahimi, 1982); (2) 1 % ruthenium red to detect negatively charged components of the colleter palisade cells (Luft, 1971); and (3) periodic acid-thiocarbohydrazide (THC)–silver proteinate (PATAg) to detect polysaccharides containing 1,2-glycol groups. For the last technique, sections were either treated with periodic acid or, to provide controls, solutions not containing periodic acid and THC (Thiéry, 1967). Sections were observed at 80 kV using a transmission electron microscope (ZEISS EM 900).

Scanning electron microscopy

The stipules were fixed, post-fixed and dehydrated as for light microscopy. The samples were subsequently critically point dried in CO_2, sputter coated with 20 nm gold, and observed with a digital scanning electron microscope (ZEISS DSEM 962).

Biochemical assays

Stipules of *Simira glaziovii* were washed in the presence of 0·1 M Tris–HCl plus 0·1 % Triton X-100, pH 8·0, to extract proteins and carbohydrates. The material was filtered through 0·45-μm Millipore filters prior to biochemical analysis. Protein determinations were performed by the method of Bradford (1976) using bovine serum albumin as a standard. The absorbance at 595 nm was measured on a UV-visible spectrophotometer. The filtrate obtained was precipitated overnight with solid ammonium sulfate (90 % saturation). The resulting precipitate was dialysed against distilled water (48 h), and recovered by freeze-drying for electrophoresis. SDS–polyacrylamide gel electrophoresis (PAGE) was performed as described by Laemmli (1970). Proteins utilized as molecular mass standards for SDS–PAGE were bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa) and α-lactoglobulin (14 kDa).

The amount of carbohydrate in the secretory fraction was evaluated by the phenol–sulfuric acid method as described by Dubois *et al*. (1956) employing glucose as standard. The absorbance at 486 nm of each fraction was measured. The average of the three absorbance determinations from each sample was used to calculate the amount of carbohydrate present in the secretions.

RESULTS

External morphology

Colleters are found on the adaxial surface of stipules of *Simira glaziovii*, *S. pikia* and *S. rubra*. In *S. glaziovii* (Fig. 1A and B), the colleters form two triangular groups that contour the base of the leaf primordia at the shoot apex (Fig. 1A, arrows). The colleters are aligned in a single row in *S. pikia* (Fig. 1C) and several rows in *S. rubra* (Figs 1D and 2C). Developing colleters appear as rounded projections on the young stipule (as shown for *S. glaziovii* in Figs 1A and 2B). Fully developed colleters are cylindrical (Figs 1B–D and 2A and C). Secretions (Figs 1B and C and 2A) cover the interior of the entire shoot apex. The secretion is viscous and becomes sticky as it dries. Secretions are yellowish for *S. glaziovii*, and colourless for *S. pikia* and *S. rubra*.

Internal morphology

Observation of thick sections of colleters showed that the colleters of the three species were the standard type (Figs 2D, 2E and 3B–D). Meristematic epidermal and subepidermal layers contribute to the morphology of these structures (Fig. 3A); as is characteristic of an emergentia. Before complete development of the colleters, the epidermal cells are difficult to differentiate from parenchyma cells (Fig. 3A). However, at maturity, the epidermal cells become more densely cytoplasmic and columnar (Fig. 3B–D).

A constriction develops at the colleter base (Fig. 3D) in the three species studied. The epidermal cells in the region of constriction are parenchyma-like in appearance. Colleters of *S. pikia* and *S. rubra* have only parenchyma cells at their centres (Fig. 3C and D). *Simira glaziovii* may have a

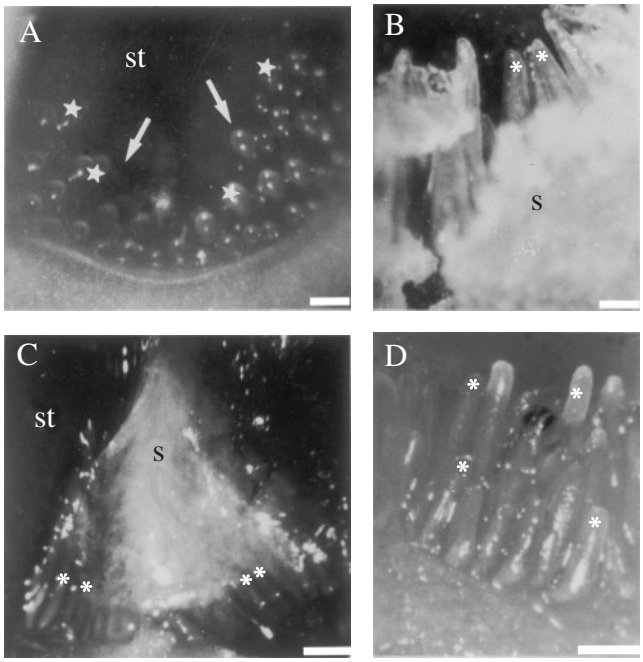

FIG. 1. Stipules detached from the shoot apex and observed with the aid of a stereomicroscope. (A and B) Colleters *of Simira glaziovii* at (A) initial stage and (B) fully developed. Note the triangular organization at the base of stipule (arrows in A). (C) Colleters of *S. pikia* and (D) colleters of *S. rubra*. Scale bars: A, C and D = 125 μm; B = 1·0 mm. Asterisks, colleters; s, secretion; st, stipule; stars, immature colleters.

vascular trace within the parenchymatic core (Fig. 3B). Epidermal cells of *S. pikia*, in contrast to the other two species, have dark-staining materials adjacent to the cell walls (Fig. 3C).

Ultrastructure of colleters

During early development of the colleters of *S. glaziovii* (Fig. 2A) epidermal (Fig. 4A) and subepidermal cells are undifferentiated. Both epidermal and subepidermal cells at this early stage of development are isodiametric, and exhibit small vacuoles, endoplasmic reticulum, Golgi apparatus, mitochondria, the nucleus and numerous electron-dense lipid bodies (Fig. 4A). As colleters develop, epidermal cells become more elongate and rectangular. At an intermediate stage of maturity, colleters become densely cytoplasmic. Vacuoles, endoplasmic reticulum, Golgi apparatus, mitochondria and the nucleus are evident (Fig. 4B). At this stage, parenchymatic cells of the core are also rapidly differentiating but differ because they have a large vacuole.

In the outer cell wall of epidermis at an intermediate stage of maturity, the cuticular layer has not fully developed a reticulated network of polysaccharides as observed by cytochemical staining. The polysaccharide portion of the cuticular layer reacts with both PATAg (specific for polysaccharides) and with ruthenium red (specific for pectins). In contrast, the lipid portion of the cuticular membrane, the cuticle proper and the matrix of the cuticular layer react with imidazole.

At maturity, the epidermal cells of the colleters in the three species studied become columnar. They have a dense cytoplasm with abundant ribosomes, a nucleus, small vacuoles, many mitochondria, endoplasmic reticulum and Golgi apparatus (Fig. 4C–G). In contrast, the parenchymatic cells have a large central vacuole. In the three species, the outer cell wall of the colleters has an lamellar layer, a cuticular membrane and cuticle proper (Fig. 4F). The cuticular membrane is reticulated (Fig. 4F), being different from the outer cell wall of the stipules that remains non-reticulated. The cuticle does not appear to rupture at maturity.

Extraplasmic spaces are found between the cell wall and the plasma membrane. Large quantities of endoplasmic reticulum are observed near the membrane (Fig. 4D–F). The Golgi apparatus is observed in all regions of these cells (Fig. 4D and E), and several vesicles are observed close to this organelle (Fig. 4G).

Several unique features were observed only in the epidermal cells of the three species. The mature epidermal cells of *S. glaziovii* showed lipidic bodies and large vesicles near the plasma membrane (Fig. 4C). In *S. pikia*, plastids are observed in the epidermal cells (Fig. 4D). In this species, the spaces between the plasma membrane and the cell wall are also filled with secretory material (Fig. 4D and E). Portions of this secretion have fibrillar structures in them (Fig. 4E). *Simira rubra* exhibits plasmodesmata between epidermal cells and the parenchymatic cells.

A few microorganisms were found embedded in the secretion closest to the outer cell wall of colleters of *S. glaziovii* (Fig. 4H) and *S. rubra*.

Biochemistry of secretion

Carbohydrates and proteins were detected in the secretion of *S. glaziovii*. Concentrations were 0·045 mg of protein mg^{-1} dry secretion and 0·2 mg of carbohydrate mg^{-1} dry secretion. Crude secretion preparations analysed by SDS–PAGE (Fig. 5) showed that the secretion from *S. glaziovii* is a mixture of proteins with molecular masses covering a range of approx. 45 to 14 kDa, with five major proteins of 45, 36, 29, 22 and 16 kDa (Fig. 5, column A). Inter- or intra-chain disulfide linkages occur in some of these proteins as demonstrated by treatment with reducing agents (Fig. 5, column B).

DISCUSSION

Simira glaziovii, *S. pikia* and *S. rubra* develop colleters at the bases of their stipules. However, the three species show a different distribution of colleters on their stipules, a triangular arrangements in *S. glaziovii*, a single row in *S. pikia*, and many rows in *S. rubra*. This arrangement of colleters on the stipule can be used as an aid in species identification. The species studied are woody taxa of the tribe Rondeletieae in the subfamily Cinchonoideae (Silva Neto, 2000).

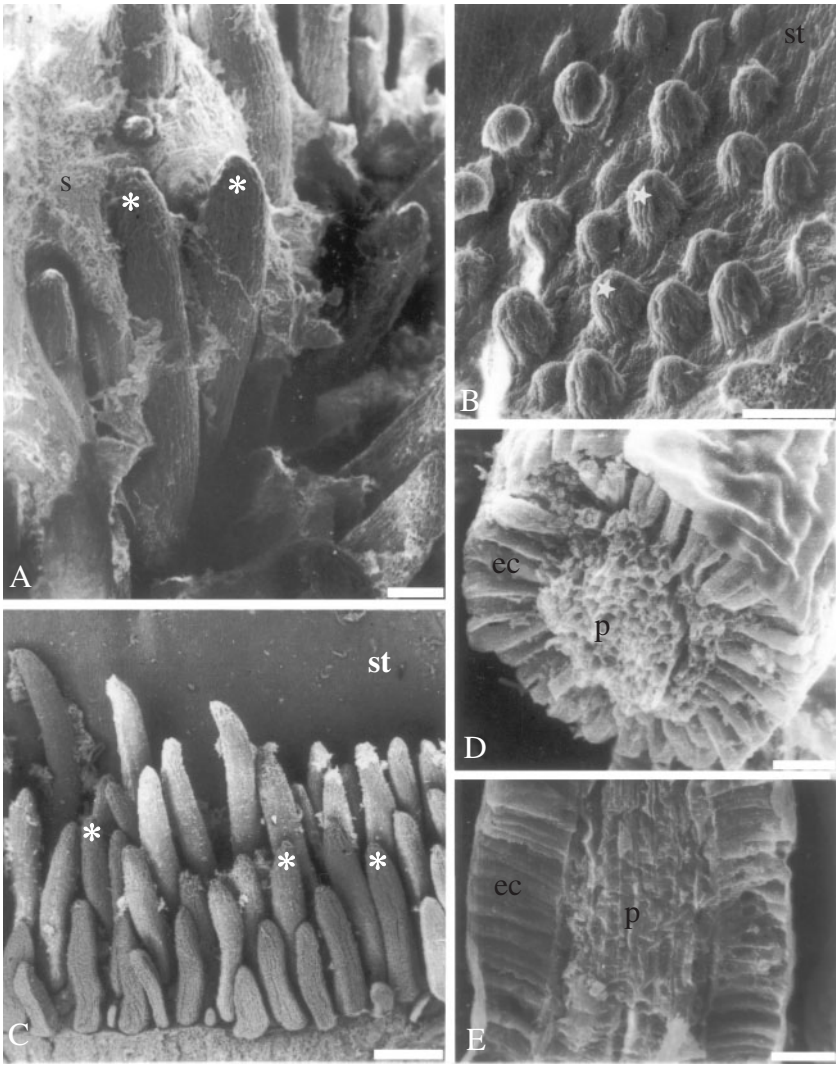

FIG. 2. Scanning electron microscopy. (A and B) An overview of the colleters of *Simira glaziovii* at (A) entirely developed and (B) initial stage; (C) an overview of the colleters of *S. rubra*; (D) cross-section of a colleter of *S. glaziovii*; and (E) longitudinal section of a colleter of *S. pikia*. Scale bars: A and B = 100 μm; C = 250 μm; D and E = 25 μm. Asterisks, colleter; stars, immature colleters; ec, epidermal cells; p, parenchyma; s, secretion; st, stipule.

Colleters of the three species are the standard type (Lersten, 1974*a*, *b*). The characters that describe the standard type, and separate it from other types, are that epidermal cells are columnar and not separated from each other. They also have a central parenchymatic axis (Lersten, 1974*b*).

The presence of a constriction or pedicel at the base of the colleters is poorly discussed in the literature. In species of *Simira* studied, colleters develop a constriction at their base. This character was observed in other rubiaceous species (Lersten, 1974*a*; Da Cunha and Vieira, 1997). In addition, the presence of chlorophylous pedicel cells was noted in the colleters of *Allamanda* (Apocynaceae; Ramayya and Bahadur, 1968).

Vascular traces, observed only in the parenchymatous core of the colleters of *S. glaziovii*, do not appear to have any importance in the activity of these secretory structures. Appezzato-da-Gloria and Estelita (2000) argued that the presence of a vascular trace is only dependent upon the proximity of these traces to the region of the colleter projection. More studies regarding the relationship between the colleters and vascularization are required, however, to establish its importance and its role in the transport of nutrients to secretory cells.

The knowledge of the colleter ultrastructure is restricted to a few species (Horner and Lersten, 1968; Dexheimer and Guenin, 1981; Miller *et al*., 1983; Durkee *et al*., 1984; Mohan and Inamdar, 1986). In all the species examined ultrastructurally, the endoplasmic reticulum occurred in perinuclear and peripheral locations in the cells. Secretory material was also observed between the cell membrane and the cell wall. In all cases, the endoplasmic reticulum and Golgi apparatus appeared to be involved in the production of mucilage (Fahn, 1988) as for *Simira*. Dexheimer and Guenin (1981) observed that, for the colleters of *Psychotria bacteriophila* (Rubiaceae), the production of mucilage protein occurred at the endoplasmic reticulum and polysaccharides on the Golgi apparatus. In contrast, mitochondria were also shown to be involved directly in mucilage production in

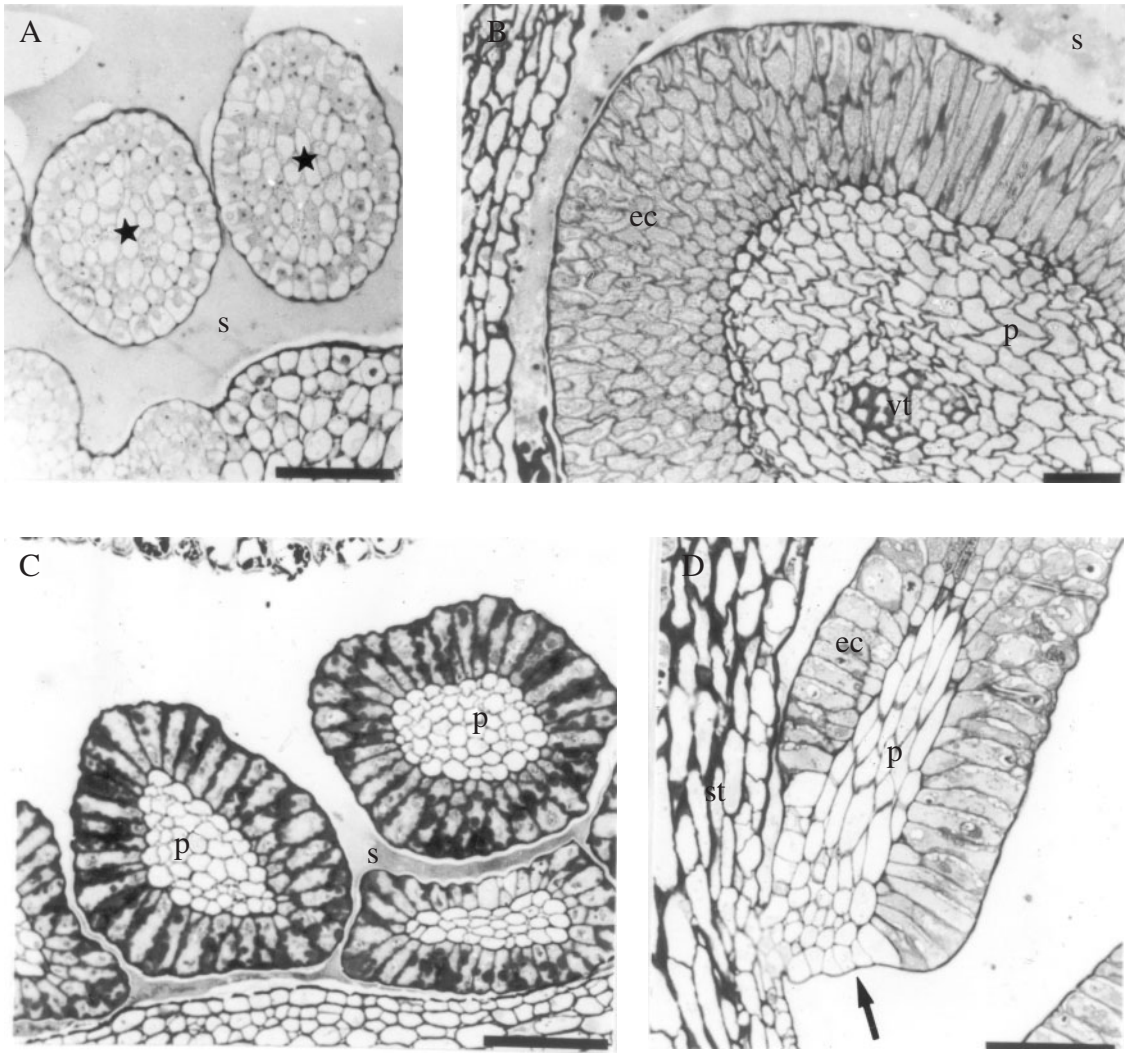

FIG. 3. Light microscopy. (A and B) Cross-sections of the colleters of *Simira glaziovii* at (A) initial stage and (B) fully developed; (C) cross-section of the colleters of *S. pikia*; and (D) longitudinal section of a colleter of *S. rubra*. Scale bars: A, B and D = 50 μm; C = 60 μm. Stars, immature colleters; arrow, constriction; ec, epidermal cells; p, parenchyma; s, secretion; st, stipule; vc, vascular trace.

the root hairs of *Sorghum* (Werker and Kislev, 1978). For *Simira* species, the presence of endoplasmic reticulum and Golgi apparatus in the periphery of the cell, next to the spaces between the cell wall and plasma membrane, suggests an involvement of these organelles in the production of secretions.

A few studies have described changes in secretory constituents during the development of secretory structures. In *S. glaziovii*, lipidic bodies were observed at matures stage of cell development. The absence of these bodies in *S. pikia* and *S. rubra* colleters may imply that observed colleters were less mature. Conversely, the presence of plastids in *S. pikia* cannot be explained in the same manner, considering that plastids may be involved in the production of secretions (Horner and Lersten, 1968; Kristen, 1976; Miller *et al*., 1983; Mohan and Inamdar, 1986). The presence of plasmodesmata in colleters of *S. rubra* was discovered for the first time for a colleter epidermal cell.

The three species studied exhibited a space between the plasma membrane and the epidermal cell wall. This space was identified as the extraplasmic space by Akers *et al*. (1978). These spaces were not found to be filled with secretory material in *S. glaziovii* and *S. rubra*. However, *S. pikia* was found to have these spaces filled with a dense secretion. These differences in secretion partitioning for different species can be found in other secretory structures (Rachmilevitz and Fahn, 1972; Zamski *et al*., 1987).

This study showed that the outer cell wall of the mature colleter developed a reticulated network of polysaccharides in its cuticular layer. In contrast, the immature cell wall does not develop this network. Although many studies report that secretions are released via cuticular rupture (Horner and Lersten, 1968; Thomas and Dave, 1989), the cuticle was not observed to rupture in colleters of *Simira*. This observation suggests an involvement of the outer cell wall in the secretion process of, for example, the

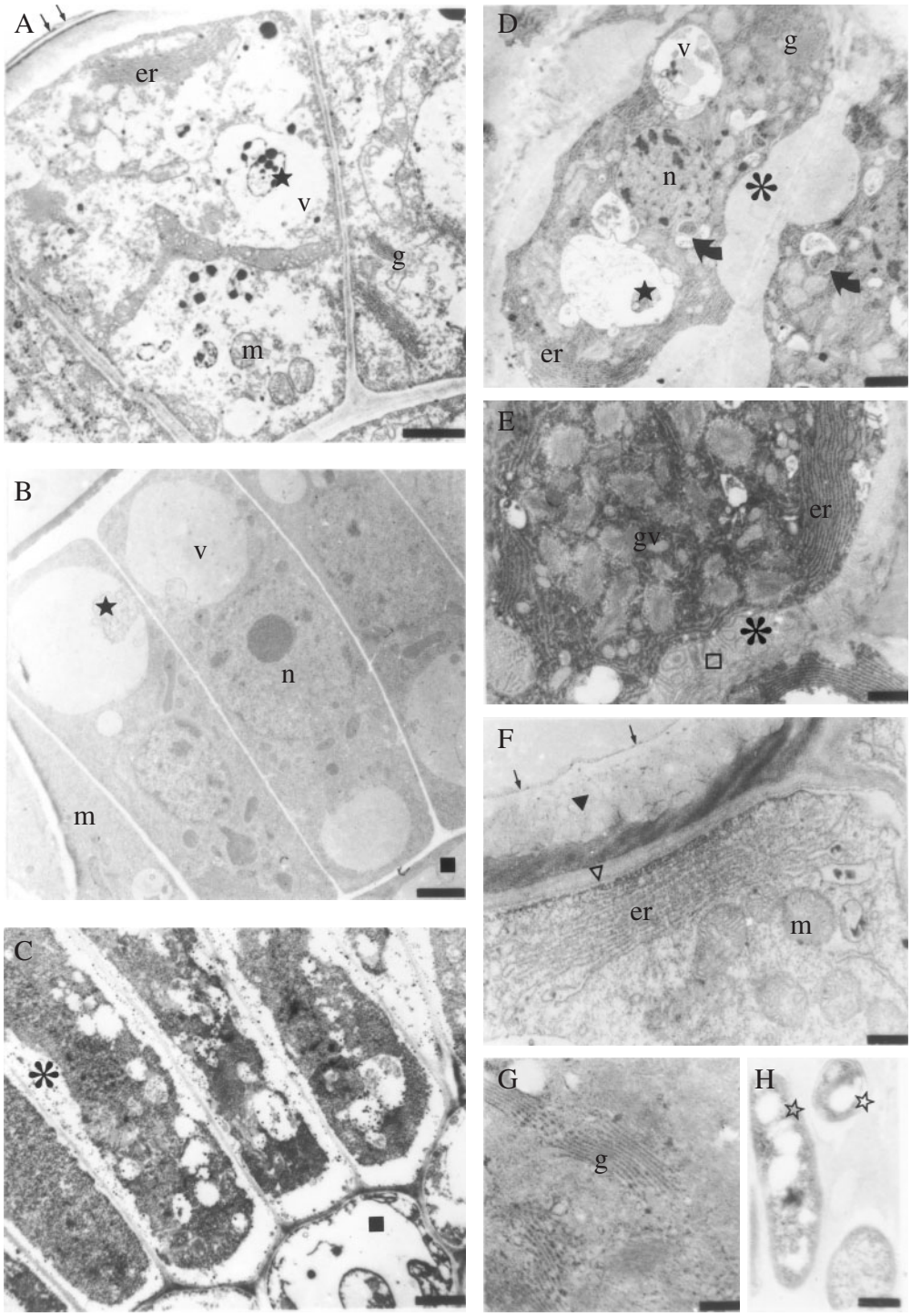

FIG. 4. Transmission electron microscopy. (A–C) Secretory cells of *Simira glaziovii* in (A) immature stage, (B) intermediate stage and (C) mature stage of development; (D and E) secretory cells and (G) Golgi apparatus of *S. pikia*; (F) outer cell wall and organelles of the secretory cell of *S. rubra*; and (H) bacteria adjacent to the outer cell wall of the colleter of *S. glaziovii*. Scale bars: A = 1·0 μm; B and C = 2·5 μm; D = 1·7 μm; E = 1·1 μm; F = 0·4 μm; G and H = 0·25 μm. Asterisks, extraplasmic space; er, endoplasmic reticulum; g, Golgi stacks; gv, Golgi vesicles; m, mitochondria; open stars, microorganisms; n, nucleus; curved arrows, plastid; square parenchymatic cell; v, vacuole; arrows, cuticle proper; triangle, cuticular membrane; open triangle, polysaccharide layer; open square, fibrilar structures; stars, debris.

trichomes of *Cannabis* (Cannabaceae). In *Cannabis*, the secretion must pass through the outer cell walls in a vesicle-like structure to exit the cell (Mahlberg and Kim, 1992; Kim and Mahlberg, 1995). In the colleters of *Simira*, these vesicle-like structures were not found in the outer cell walls.

The general properties of colleters as defensive structures have been described for several species (Ramayya and

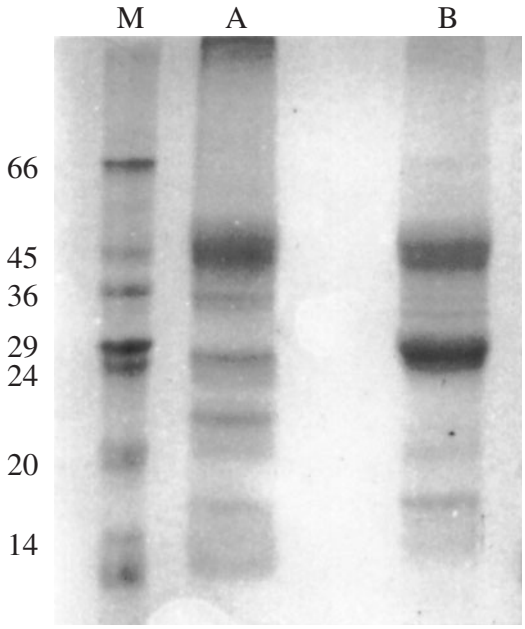

FIG. 5. SDS–polyacrylamide gel electrophoresis of proteins from *Simira glaziovii* secretion. A, Ammonium sulfate fraction; B, ammonium sulfate fraction treated with β-mercaptoethanol; M, markers (kDa).

Bahadur, 1968; Williams *et al.,* 1982, Thomas and Dave, 1990). The biological significance of the colleter secretion is enhancing protection of the meristematic tissues (Robbrecht, 1988). In addition, the main function of secretions in plants is thought to be a defense mechanism against herbivores and microorganisms (Zalucki *et al*., 2001; Cruz *et al*., 2002). Exudates, for example, are known to contain several proteins related to plant defense, including chitinases, polyphenol oxidases and β-1,3-glucanases (Subroto *et al*., 1996; Wititsuwannakul *et al*., 2002; Azarkan *et al*., 2003). Our present biochemical analysis shows the presence of proteins and carbohydrates in secretions from *S. glaziovii*. These proteins are possibly associated with defense mechanisms against microorganisms. Further studies are required to determine the role of these proteins in plant protection.

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