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ORIGINAL ARTICLE

Ultrastructure and secretion of glandular trichomes in species of subtribe Cajaninae Benth (Leguminosae, Phaseoleae)

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Abstract

The subtribe Cajaninae of papilionoid legumes has a pantropical distribution and comprises approximately 490 species. These species have diversified throughout dry environments where there are high temperatures and strong light. The subtribe stands out because all its representatives have vesicular glands. In addition, bulbous-based and capitate trichomes are important secretory structures present in all genera of the Cajaninae. We analyzed the ultrastructure and histochemistry of these glandular trichome types in leaflets of the three species of the subtribe. Using transmission electron microscopy and histochemical analyses, we link the glandular secretions to subcellular structures. We here report for the first time the type of exudate and ultrastructure of the glands of subtribe Cajaninae. Terpenoids and phenolics were confirmed by histochemistry tests, and we observed that the organelles responsible for biosynthesis of oils are the most representative in these glands. Each glandular trichome showed particular ultrastructural features compatible with the compounds produced. We suggest that these glandular trichomes, with their respective exudates, act in defense against herbivory and against possible damage by ultraviolet radiation.

Keywords Cajaninae glands · Fabaceae · Phenolic compounds · Terpenoids

Introduction

Plants produce a wide variety of secretions, from aqueous solutions rich in salts, amino acids, and sugars to more complex mixtures consisting of primary metabolites (e.g., proteins, polysaccharides) or primary and secondary metabolites (e.g., terpenoids, phenylpropanoids, and alkaloids). Many exudates contain biologically active molecules that contribute to the evolutionary success of the species that produce them (Ascensão 2007) can help in the interactions of plants with their surrounding environment (Fahn 1979, 2002).

Secretions are produced by isolated cells or specialized multicellular structures that are extremely variable in morphology (Fahn 1979; Evert 2006; Ascensão 2007). In Leguminosae, many types of secretory structures in vegetative organs have been recorded, including glandular trichomes, secretory epidermal cells, extrafloral nectaries, idioblasts, canals, and cavities (Solereder 1908; Metcalfe and Chalk 1950, 1979, 1983; Pyykko 1966; Fahn 1979; Gregory and Baas 1989; Lersten and Curtis 1993, 1994, 1995, 1996; Sartori and Tozzi 2002; Fortuna-Perez et al. 2012; Pereira et al. 2018).

Focusing on glandular trichomes, the Leguminosae displays a wide diversity of these structures, including peltate glands in *Indigofera* L and *Centrolobium* Mart ex Benth (Metcalfe and

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Chalk 1950; Matos and Paiva 2012), cavitated secretory trichomes in *Bauhinia* L. *s.s.* (Marinho et al. 2015), *Hoffmannseggia* Cav., *Caesalpinia* L. *s.l.* (Lersten and Curtis 1994, 1996), and *Indigofera* L. (Marquiafável et al. 2009), uniseriate filiform capitate hairs in *Alysicarpus rugosus* (Willd.) DC., uniseriate filiform hairs in *Melilotus albus* Medik., uniseriate prickle-like hairs in *Vigna cylindrica* (L.) Skeels (Leelavathi and Ramayya 1983), and especially, capitate trichomes, bulbous-based trichomes, and vesicular glands in representatives of subtribe Cajaninae (Lackey 1978; Vargas et al. in press).

Earlier published reports about the types of secretory structures occurring in Cajaninae maintained that the bulbous-based trichomes and vesicular glands are typical secretory structures of the subtribe (Debold 1892; Lackey 1978; Vargas et al. 2015; Vargas et al. in press). The subtribe Cajaninae has a pantropical distribution, and its taxa are found in grasslands, seasonally dry tropical forests, semi-arid areas, disturbed areas, and fire-prone environments (Schrire 2005). Cajaninae comprises approximately 490 species in ten genera, with the two genera *Eriosema* (DC.) Desv. and *Rhynchosia* Lour. being the most speciose, with 150 and 230 species, respectively (Schrire 2005). These two genera possess the African and American continent as the main centers of diversity (Grear 1970).

Considering these micromorphological structures, the capitate trichomes consist of a short, single basal cell, usually rectangular to square in shape, a unicellular to absent stalk, and a clavate head (Vargas et al. in press); while bulbous-based trichomes are composed of one to two basal cells supporting a group of cells that form the bulbous base, a number of apically elongated cells, and slightly rounded terminal cells (Solereder 1908; Vargas et al. 2015). The vesicular glands have a trapezoidal to square basal cell (Vargas et al. in press), a narrow, short stalk supporting a large and spherical head surrounded by an amber-colored exudate. These vesicular glands are frequently inserted into an epidermal depression, (Solereder 1908; Lackey 1978; Lackey 1981). Uphof (1962) described how in the vesicular glands all cells of the head break down to form the yellow glandular fluid. These various glandular trichomes are extensively distributed throughout the leaflets of taxa in the Cajaninae, and provide important diagnostic value (Vargas et al. in press). Capitate trichomes are widely encountered in tribe Phaseoleae (Lackey 1978), and in subtribe Cajaninae, they occur in some species and their presence or absence can be used as a diagnostic character (Vargas et al. in press). Bulbous-based trichomes also occur in all Cajaninae genera but are not an exclusive characteristic of the subtribe (Leelavathi and Ramayya 1983; Vargas et al. in press). In almost all Cajaninae taxa, bulbous-based trichomes are less abundant than vesicular glands; nevertheless, they serve as a diagnostic character at infra-generic taxonomic rank in the subtribe (Vargas et al. 2015; Vargas et al. in press). Vesicular glands are exclusively and abundantly distributed among Cajaninae taxa and they are a unifying character of the subtribe (Vargas et al. in press), facilitating the prompt identification of any member of Cajaninae.

Given the anatomical variation of these glandular trichomes in Cajaninae and their taxonomic diagnostic value at infra-tribal and infra-generic levels, we considered that a combination of ultrastructural and histochemical analyses might provide support in understanding the functions of each gland type. Here, we investigate and compare the histochemistry and ultrastructure of capitate trichomes, bulbous-based trichomes, and vesicular glands in a search for similarities or differences among them and we consider the potential function of these trichomes. *Rhynchosia minima* (L.) DC., *Eriosema rufum* (Kunth) G. Don, and *Eriosema simplicifolium* (DC.) G. Don were selected as model species for this study.

Materials and methods

Plant material

Samples of young leaflets of *R. minima*, *E. rufum*, and *E. simplicifolium* were collected from wild populations occurring in the Cerrado (Brazilian savanna) and forest vegetation of the states of São Paulo and Minas Gerais, Brazil (Table 1). Voucher specimens were deposited in the Irina Delanova Gemtchújnicov Herbarium (BOTU) in the Institute of Biosciences of UNESP, Botucatu, São Paulo, Brazil. The three types of trichomes most widely distributed in the Cajaninae leaflets (according to Vargas et al. in press) were analyzed in their secretory phase (= substance release) in three individuals of each species.

Histochemical tests

Fresh samples of leaflets of *R. minima*, *E. rufum*, and *E. simplicifolium* or those fixed with formalin–acetic acid–alcohol (FAA) for 24 h and buffered formalin for 48 h (Lillie 1965) were hand-cut and subjected to the following treatments for histochemical analyses: Sudan IV (Pearse 1980) for total lipids, Nadi reagent (David and Carde 1964) for terpenoids, vanillin-hydrochloric acid (Mace and Howell 1974) for tannins, ferric chloride (Johansen 1940) for phenolic compounds, Dragendorff reagent (Svedsen and Verpoort 1983) for alkaloids, bromophenol blue (Mazia et al. 1953) for total proteins, tannic acid–ferric (Pizollato 1977) for mucilage, and Nile blue (Cain 1947) for acidic lipids. The control samples were tested according to the specifications of each test. Micrographs were obtained using a light microscope (Olympus BX 41) linked to a digital camera in the Laboratory of Plant Anatomy of UNESP—Botucatu (LAPAV).

Table 1 Voucher information for the studied taxa

Taxa	Locality	Vegetation	Collector	Herbarium
Eriosema simplicifolium (DC.) G. Don	Botucatu, São Paulo, Brazil	Cerrado, open grassland in rocky areas	W Vargas and DP Seixas 76; W.Vargas 81 and 82	BOTU
Rhynchosia minima (L.) DC.	Botucatu, São Paulo, Brazil	Cerrado, edge of dry forest	W Vargas 71, 83, 84	BOTU
Eriosema rufum (Kunth) G. Don var. *rufum*	Corinto, Minas Gerais, Brazil	"campos cerrados" and pastures on sandy soils	W Vargas et al. 80, 85, 86	BOTU

Transmission electron microscopy

For transmission electron microscopy (TEM), samples of the middle portion of leaflets from *R. minima*, *E. rufum*, and *E. simplicifolium* were removed and fixed for 24 h in 2.5% glutaraldehyde in 0.1 mol L^{-1} phosphate buffer (pH 7.3), after which the materials were post-fixed with 1% osmium tetroxide aqueous solution in the same buffer for 1 h at 25 °C. The samples were then dehydrated in an acetone series and embedded in Araldite resin (Machado et al. 2006). Ultrathin sections (70 nm) were stained with uranyl acetate and lead citrate (Reynolds 1963) and then examined with a transmission electron microscope (FEI Tecnai) at 80 kV at the Electron Microscopy Center of the Institute of Biosciences of Botucatu, UNESP.

Results

Capitate trichomes and vesicular glands occurred on both leaflet surfaces of all studied taxa, except for *E. rufum* that the capitate trichomes occurred only on the adaxial surface. Bulbous-based trichomes were present in *R. minima* and *E. rufum* on the abaxial surface (Table 2).

Through histochemical analysis, we verified that total lipids occurred in all glandular trichomes observed (image for vesicular gland Fig. 1a, b). Essential oils occurred only in bulbous-based trichomes (Fig. 1c) and acidic lipids in bulbous-based trichomes (Fig. 1d) and capitate trichomes. Phenolic compounds occurred in bulbous-based trichomes (Fig. 1e) and vesicular glands (Fig. 1f) only in *R. minima*. Proteins were detected in capitate trichomes and vesicular gland. Alkaloids, polysaccharides (mucilage), and tannins were not detected. The positive reaction was detected in different sites of these trichomes (Table 3; Fig. 1).

The capitate trichomes were composed of a basal cell and a multicellular head covered with a continuous thick cuticle (Fig. 2a). The basal cell was projected above the other cells of the epidermis and its side walls were covered by a thicker cuticle (Fig. 2a). The basal cell had a thin tangential wall, a parietal voluminous nucleus, a large central vacuole, and a reduced cytoplasm (Fig. 2a). Rounded plastids, devoid thylakoid and with oil drops, mitochondria with well-developed cristae, and a rough endoplasmic reticulum characterized the cytoplasm of this cell (Fig. 2b). The head cells had a square or rectangular shape and exhibited thin walls, a large central nucleus, vacuoles of different sizes, and abundant cytoplasm (Fig. 2c, d). In the central region of the head cells, where more than two cells are joined, and in the distal portion of anticlinal walls in contact with cuticle, the middle lamella was more electron-dense and thickened (Fig. 2c, d). In these regions, the middle lamella showed signs of dissolution (Fig. 2d, e) and accumulation of flocculated material was frequently observed at these sites. The cytoplasm of the head cells contained free ribosomes, polyribosomes, plastids, a rough endoplasmic reticulum (RER), mitochondria with developed cristae, and Golgi bodies (Fig. 2f). The plastids were polymorphic, devoid thylakoids, and had starch grain or lipid inclusions (Figs. 2f and 3a, b). The rough endoplasmic reticulum was extensive, and some profiles exhibited dilation (Fig. 3b). The Golgi bodies were composed of two to four elongated cisternae with few vesicles in the ends (Fig. 3b). Coated vesicles from RER occurred in the peripheral cytoplasm and juxtaposed to the plasmalemma, and showed signs of fusion with this membrane (Fig. 3c), clustered inside the multivesicular bodies located near the plasmodesmata, and scattered in the

Table 2 Distribution of secretory structures in three species of Cajaninae

Species	Vesicular glands		Bulbous-based trichomes		Capitate trichomes	
	Ad	Ab	Ad	Ab	Ad	Ab
Eriosema simplicifolium (DC.) G. Don	+	+	−	−	+	+
Rhynchosia minima (L.) DC.	+	+	−	+	+	+
Eriosema rufum (Kunth) G. Don var. *rufum*	+	+	−	+	+	−

+ positive, − negative, *Ad* adaxial, *Ab* abaxial

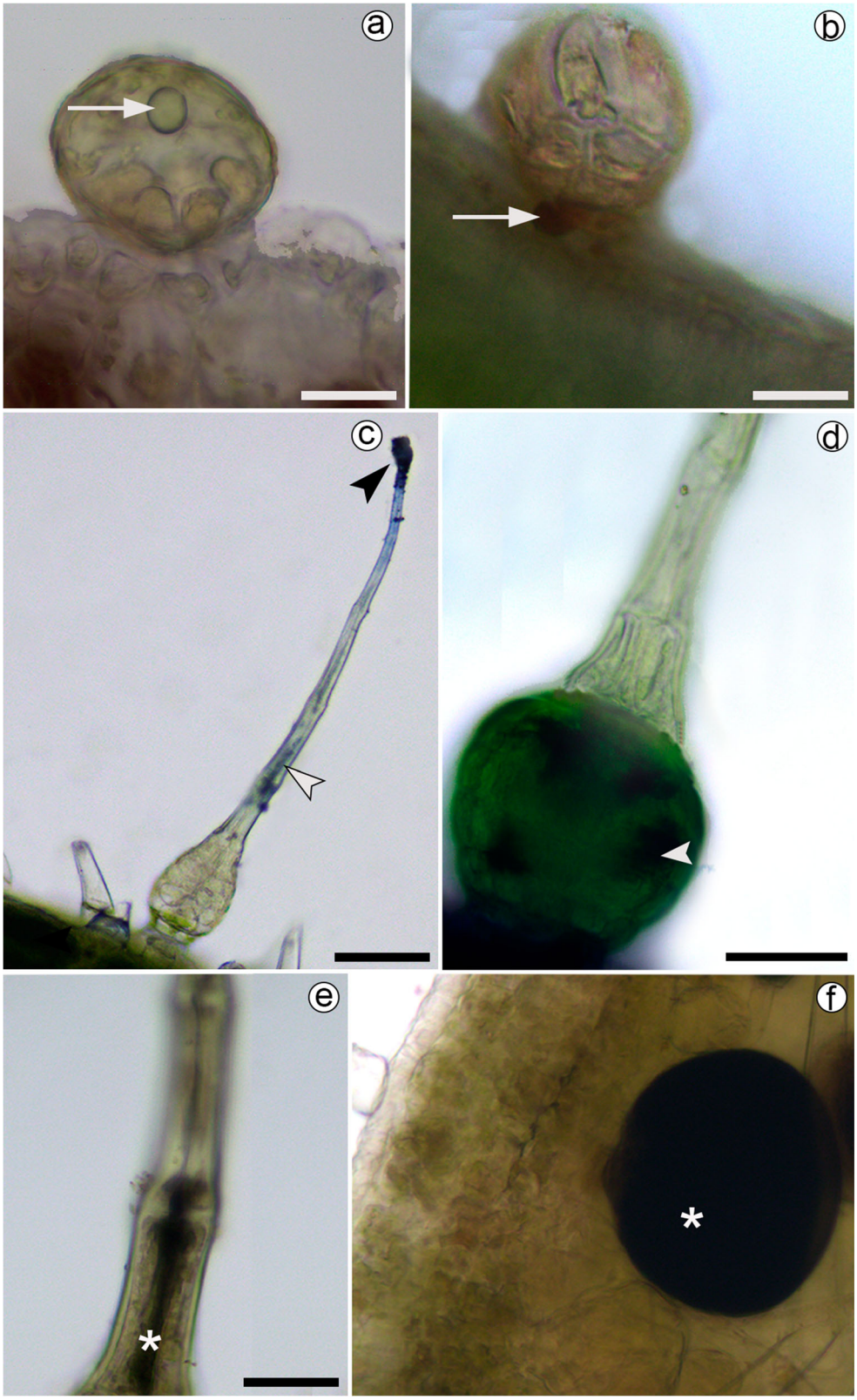


Fig. 1 Micrographs of the histochemical tests in Cajaninae glands in *E. simplicifolium* (**a**, **b**), *E. rufum* (**c**), and *R. minima* (**d**–**f**). **a** Oil drop (white arrow) identified by the histochemical Sudan IV test in central cavity of vesicular glands. **b** Lipid (white arrow) identified by the histochemical Sudan IV test in stalk of vesicular gland. **c** Essential oils in a terminal cell (black arrowhead) and proximal cells (white arrowhead) in the neck region identified by the histochemical Nadi test of bulbous-based trichomes. **d** Acidic lipids (white arrowhead) identified by the histochemical Nile blue test in the cells of the bulbous region of bulbous-based trichomes. **e** Phenolic compounds (white asterisk) identified by the histochemical ferric chloride test in proximal cells in the neck region of bulbous-based trichomes Phenolic compounds identified by the histochemical ferric chloride test in proximal cells in the neck region (white asterisk) of bulbous-based trichomes **f** and vesicular glands. *Scale bars* **a**–**f** = 20 μm

cytosol (Fig. 3d). In these cells, the anticlinal walls had variable electron density with light areas interspersed with dark areas (Fig. 3a, b), having a loose aspect with gaps in some regions (Fig. 3e). In the distal region of the head, the cells showed RER proliferated adjacent to plasmalemma and dark materials accumulated in periplasmic spaces along the outer

Table 3 Histochemical analysis in reaction sites in each cell of the secretory trichomes of three species of Cajaninae

Type of glandular trichomes		Capitate trichome		Bulbous-based trichome				Vesicular glands			
Histochemistry test	Compounds	Reaction site									
		Basal cell	Head cells	Basal cell	Bulb cells	Neck cells	Apical cell	Basal cell	Stalk cell	Head cells
Sudan red	Total lipids	Lateral walls	Lateral walls; cuticle	Lateral walls	Lateral walls; cuticle	Lateral walls; cuticle	Lateral walls; cuticle	Lateral walls; protoplasm	Lateral walls; cuticle	Lateral walls; cuticle; subcuticular space; central cavity
Nile blue	Acidic lipids	Protoplast	Protoplast	–	Protoplast	–	–	–	–	–
Ferric chloride	Phenolic compounds	–	–	–	*Protoplast	*Intercellular space	–	*Protoplast	*Protoplast	*Protoplast
Bromophenol blue	Total proteins	Protoplast	Protoplast	–	–	–	–	Protoplast	Protoplast	Protoplast
Nadi reagent	Terpenes	–	–	–	–	Protoplast; intercellular space	Protoplast	–	–	–

– absence, *only in *Rhynchosia minima*

periclinal walls (Fig. 3f). Dark material was seen also in the cell wall, inside the cuticular layer, and on the cuticle (Fig. 3g). The outer periclinal cell wall had a reticulated appearance and the cuticle presented a large cuticular layer with electron-dense ramifications that do not reach the proper cuticle (Fig. 3h).

The bulbous-based trichomes were composed of one to two basal cells, a 1-celled stalk (or this absent), a multicellular bulbous region, and a narrow-tapered neck region with a variable number of cells axially elongated and ending in a terminal swollen spathulate cell (Fig. 1c). The trichome was covered by a thick cuticle that gradually increases in thickness towards the base (Fig. 4a, b) and apex (Fig. 5d–f). Lipid inclusions were observed in the lateral walls of the cells of the bulbous region (Fig. 4b). The base was formed by two epidermal cells (proximal and distal) that were joined by very thin tangential walls (Fig. 4c) rich in plasmodesmata (Fig. 4d). The proximal cell was larger and characterized by a rounded shape, a large central vacuole, and reduced cytoplasm (Fig. 4a, c). Extensive RER, globular mitochondria, and ellipsoidal chloroplasts with oval starch grains, plastoglobules, and a peripheral reticulum occurred in this cell (Fig. 4d). The distal cell had a lenticular form, reduced size, vacuoles with different sizes, a larger nucleus, and abundant cytoplasm (Fig. 4c). The tangential wall between the distal cell and the cells of the bulb was relatively thicker and denser (Fig. 4c, d). The first cell of the bulbous region was rectangular, and the tangential walls joined to other bulb cells were thicker and denser (Fig. 4a, e). The other cells of the bulbous region were square in shape, thin-walled, and differed in relation to vacuole development depending of their position in the swollen base (Fig. 4a). Larger vacuoles and a reduced cytoplasm occurred in the cells of the "bulb" periphery (Fig. 4a), while the inner cells had a voluminous nucleus, few small vacuoles, and an abundant cytoplasm (Fig. 4e). The vacuoles were translucent and contained fibrillar material (Fig. 4f). Smooth and rough endoplasmic reticulum, mitochondria, plastids with starch grains, signs of degradation and osmiophilic globules, and vesicles occurred in the cytoplasm of the inner cells (Fig. 4f). The vesicles occurred mainly in peripheral cytoplasm or were juxtaposed to plasmalemma (Fig. 4f). The middle lamella in the corner of the inner cells became swollen and progressively showed signs of dissolution, giving rise to intercellular spaces (Fig. 4g). In these cells, the plasmalemma was sinuous in outline and dark material occurred in the periplasmic space, the cell walls and intercellular spaces (Fig. 4g, h). The dissolution of the middle lamella progressed along the entire length of the cell and formed large tubular spaces between the cells. Accumulation of heterogeneous material was observed in these intercellular spaces (Figs. 4h and 5a). In the cytoplasm of all cells of the bulbous region, oil drops occurred scattered in the cytoplasm, in contact with the tonoplast (Fig. 5b), and inside the plastids (Fig. 5c). The vacuoles contained lipid

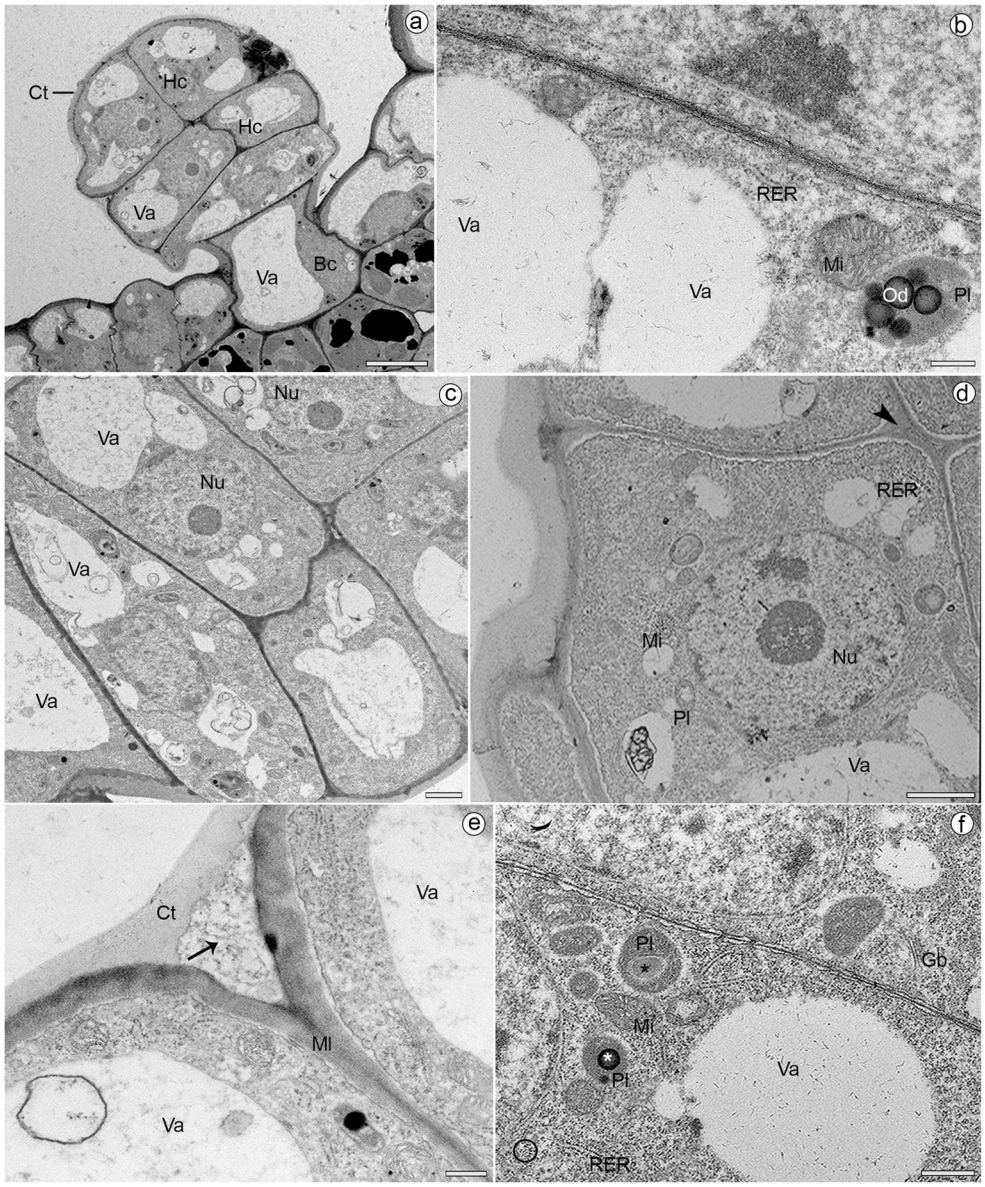


Fig. 2 TEM micrographs of capitate trichomes in *Rhynchosia minima* (**a**–**c**, **e**), *Eriosema rufum* (**f**), and *Eriosema simplicifolium* (**d**). **a** Overview of a trichome with basal cell and multicellular head covered with a thick cuticle; basal cell project above the other cell of the epidermis, exhibiting a thin tangential wall, voluminous nucleus, and large vacuoles. **b** Basal cells with rounded plastids, devoid thylakoid and with oil drops, mitochondria with well-developed cristae, and rough endoplasmic reticulum. *Head cells* (**c**–**f**). **c** Cells with square or rectangular shape and exhibiting thin walls, large and central nucleus, vacuoles of different sizes, and abundant cytoplasm. **d** Rough endoplasmic reticulum (RER), plastids, and mitochondria with developed cristae; cells in the distal portion with middle lamella more electron-dense and thick (black arrowhead). **e** Middle lamella showing signs of dissolution and accumulation of flocculated material (black arrow). **f** Cytoplasm with free ribosomes, polyribosomes (black circle), plastids with starch grain (black asterisk) and lipid inclusion (white asterisk), rough endoplasmic reticulum, mitochondria, and Golgi bodies. Bc basal cell, Ct cuticle, Gb Golgi bodies, Hc head cell, Mi mitochondria, Ml middle lamella, Nu nucleus, Od oil drop, Pl plastids, RER rough endoplasmic reticulum, Va vacuole. *Scale bars* **a** = 5 μm; **b**, **f** = 500 nm; **c**–**e** = 2 μm

inclusions and membranous material (Fig. 5b). The cell walls presented an inner reticulate layer and an outer fibrous layer, with fibrils protruding into the cuticle (Fig. 5c). In the neck region, the proximal cells had larger vacuoles and reduced cytoplasm (Fig. 5d), while distal cells had abundant cytoplasm and poorly developed vacuoles, some of them filled with multilamellar bodies (Fig. 5e). Accumulation of dense material occurred in the periplasmic and intercellular spaces, cells walls, and on the cuticle (Fig. 5e). The terminal cell of the neck region exhibited a poly-lamellate outer wall, and there was an evident transversal line of breakage between this cell and the underlying neck cells (Fig. 5f). Wall ingrowths covered with plasmalemma, characterizing labyrinthic walls, occurred in all extensions of the terminal cell of the neck region;

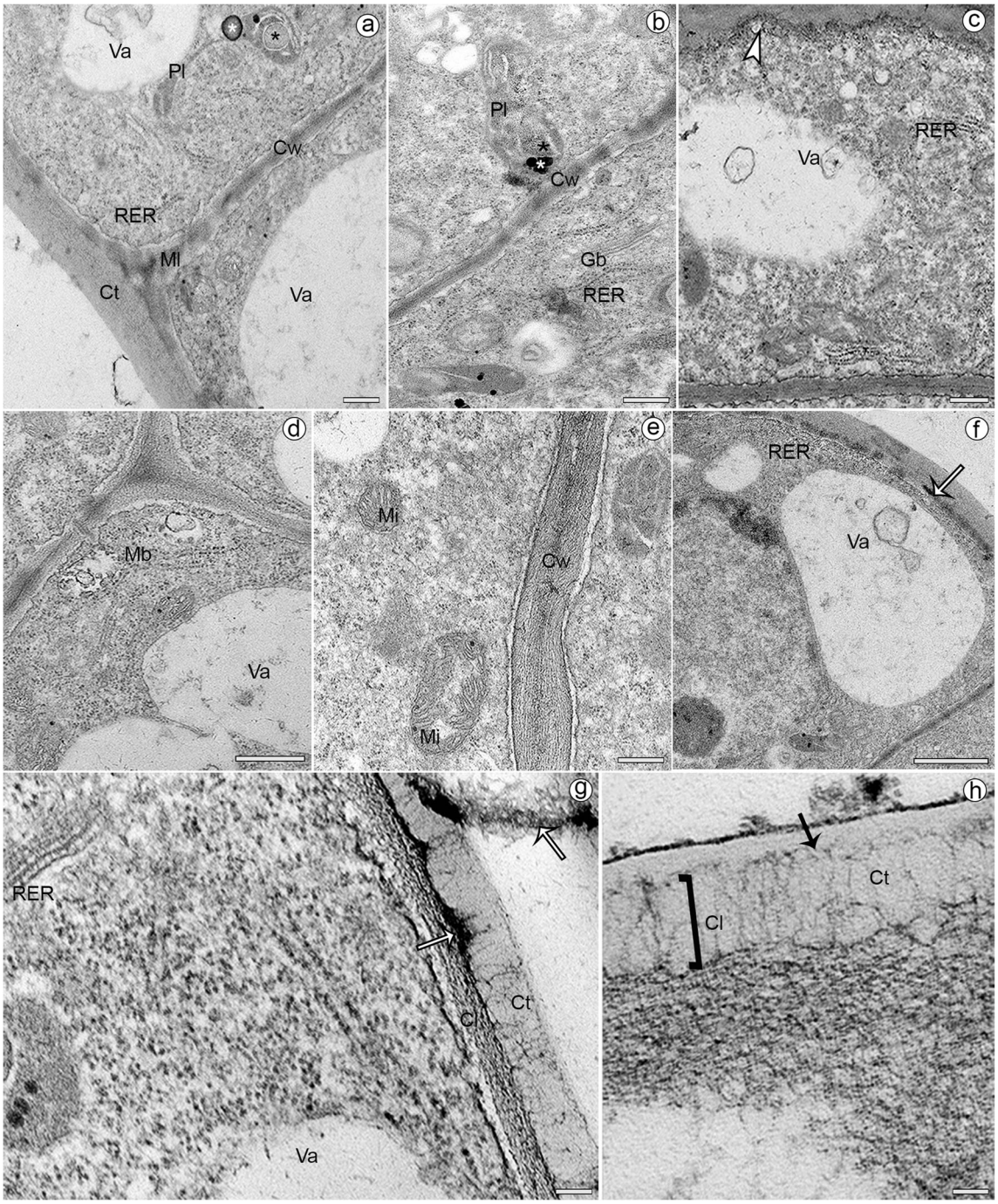
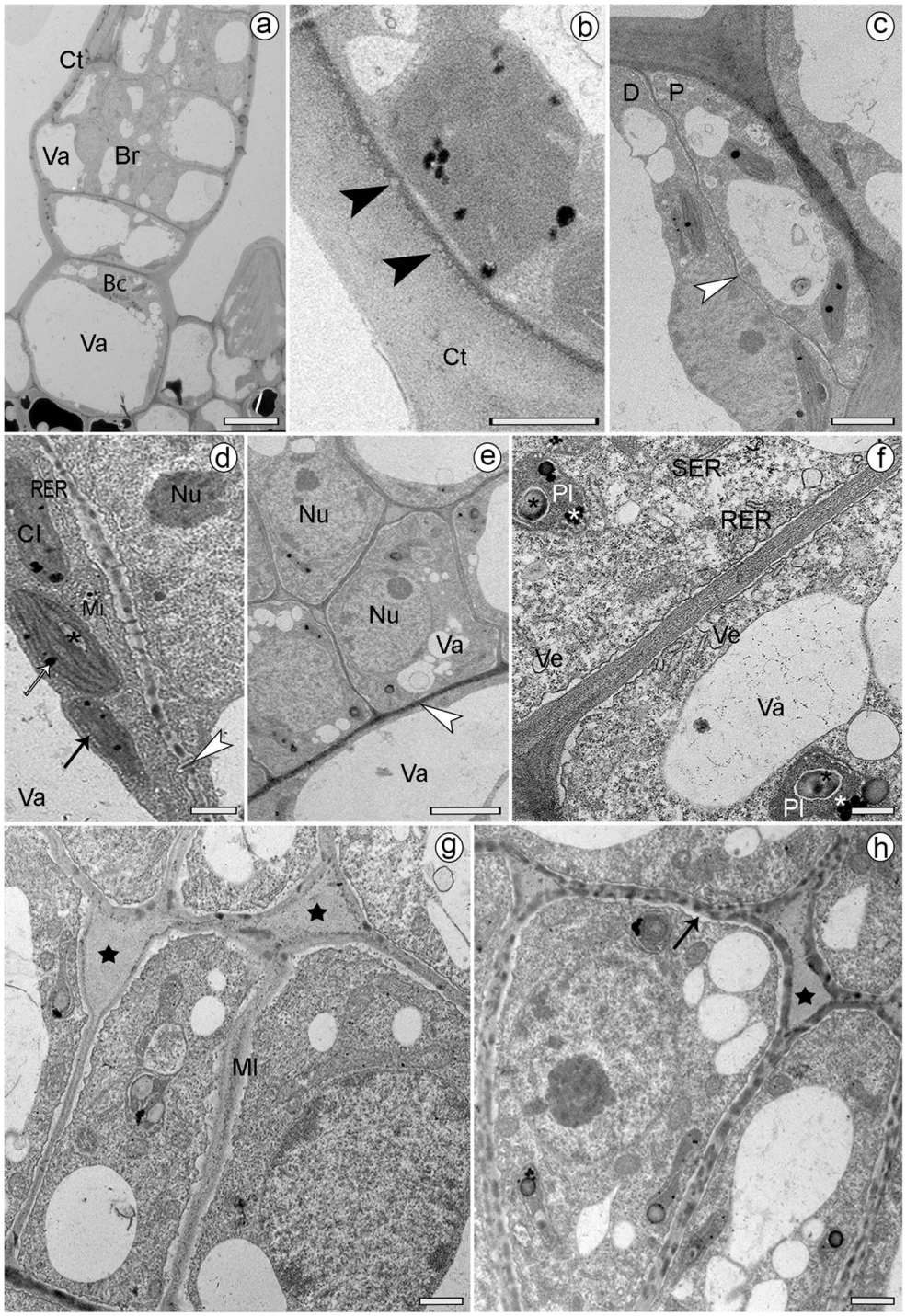


Fig. 3 TEM micrographs of the head cells of the capitate trichomes in *Rhynchosia minima* (**b**, **f**), *Eriosema rufum* (**a**, **c**–**e**), and *Eriosema simplicifolium* (**g**–**h**). **a** Anticlinal walls with variable electron density, polymorphic plastids devoid thylakoids, and with starch grains (black asterisk) and lipid inclusion (white asterisk). **b** Anticlinal walls with variable electron density, Golgi bodies with two to four cisternae, rough endoplasmic reticulum (RER), and polymorphic plastids with starch grain (black asterisk) and lipid inclusion (white asterisk). **c** Coated vesicles (white arrowhead) from RER in the peripheral cytoplasm and juxtaposed to the plasmalemma with signals of fusion with this membrane. **d** Multivesicular bodies located near the plasmodesmata and scattered in the cytosol. **e** Anticlinal walls with variable electron density exhibiting loose aspect, and mitochondria with well-developed cristae. **f** Cells in distal region showing RER adjacent to plasmalemma and dark materials (white arrow) accumulated in periplasmic space in outer periclinal walls. **g** Dark material (white arrow) in cell wall, inside cuticular layer, and on the cuticle. **h** Outer periclinal cell with reticulated aspect and cuticle exhibiting a large cuticular layer with electron-dense ramifications that do not reach the proper cuticle (black arrow). Cl cuticular layer, Ct cuticle, Cw cell wall, Gb Golgi bodies, Mb multivesicular bodies, Mi mitochondria, Ml middle lamella, Pl plastids, RER rough endoplasmic reticulum, Va vacuole. *Scale bars* **a**–**b**, **d**–**f** = 500 nm; **c** = 1 μm; **g** = 200 nm; **h** = 100 nm



microtubules arranged perpendicularly to the cell wall were common in this cell (Fig. 5g).

The vesicular glands consisted of a trapezoidal basal cell, a 1-celled stalk (or this absent), and a multicellular head with a central cavity (Fig. 6a). Frequently, this gland was inserted in an epidermal depression (Fig. 6a) and was covered with a thick continuous cuticle (Fig. 6c) that detached to form small subcuticular spaces (Fig. 6l). The basal cell had vacuoles of different sizes and abundant cytoplasm with mitochondria, and smooth endoplasmic reticulum (SER) (Fig. 6b). The stalk cell contained few small vacuoles, a dense cytoplasm with abundant mitochondria having well-developed cristae, RER, plastids with prominent starch grains, and scattered oil drops (Fig. 6b). The tangential wall was thicker and had numerous plasmodesmata connecting the basal and stalk cells (Fig. 6b). The central cavity developed by separation of the adjoining primary cell walls due to middle lamella dissolution (Fig. 6c, d), this a schizogenous process. The schizogenesis started in the corner of the inner head

◀ **Fig. 4** Transmission electron micrographs (TEMs) of the bulbous-based trichomes in *Rhynchosia minima*. **a** Overview of the basal and bulbous region cells covered with thick cuticle; the basal cells are composed by proximal and distal cell, the proximal cell have large vacuole; the cells of the periphery of the bulbous regions have large vacuoles and reduced cytoplasm, while the inner cell of the bulbous regions has small vacuoles and abundant cytoplasm. **b** Lipid inclusions (black arrowhead) in the lateral walls of the cells of the bulbous region. *Basal cells* (**c**, **d**). **c** Detail of the thin tangential wall (white arrowhead) in the proximal and distal cell. **d** The proximal cell contains vacuole, rough endoplasmic reticulum (RER), mitochondria, and chloroplasts with starch grain (asterisk), plastoglobules (white arrow), and peripheral reticulum (black arrow); the distal cell contains larger nucleus, and abundant cytoplasm; plasmodesmata (white arrowhead) between proximal and distal cells. *Bulbous region* (**e**–**h**) **e** The rectangular first cell, with thick wall (white arrowhead), and large vacuole; the inner cells with square shape, thin-walled, and have voluminous nucleus, few and small vacuoles, and abundant cytoplasm. **f** Inner cells with smooth and rough endoplasmic reticulum, plastids with starch grains (black asterisk) and osmiophilic globules (white asterisk), and vesicles in peripheral cytoplasm and near plasmalemma. **g** Inner cells with middle lamella becoming to dissolve and forming intercellular spaces (black stars). **h** Dark materials in periplasmic space (black arrow), cell walls, and intercellular space (black star) in inner cells. Bc basal cells, Br bulbous region, Cl chloroplasts, Ct cuticle, D distal cell, Mi mitochondria, Ml middle lamella, Nu nucleus, P proximal cells, Pl plastids, RER rough endoplasmic reticulum, SER smooth endoplasmic reticulum, Va vacuole, Ve vesicles. *Scale bars* **a**, **b**, **d** = 5 μm; **c**, **e** = 500 nm; **f**, **g** = 1 μm; **h** = 2 μm

cells, advanced to other wall regions (Fig. 6e), and then reached the cuticle (Fig. 6f), resulting in the displacement of the head cells towards the periphery of the gland and in the formation of a broad lumen (Fig. 6g). Polyribosomes, rounded plastids devoid of thylakoids and containing oil drops (Fig. 6h), mitochondria with well-developed cristae, extensive RER, proliferate SER, and numerous vesicles occurred in the cytoplasm of the head cells (Fig. 6i). Oil drops occurred scattered in the cytosol (Fig. 6h), in the peripheral cytoplasm near the plasmalemma facing both the central cavity (Fig. 6j) and the outer periclinal wall (Fig. 6k), and inside the periplasmic space (Fig. 6k).

Discussion

This study represents the first detailed ultrastructural and histochemical analyses of glandular trichomes of subtribe Cajaninae. The relationship between gland histochemistry, subcellular structures, and the mechanisms of secretion for the three major gland types encountered in subtribe Cajaninae (capitate trichomes, bulbous-based trichomes, and vesicular glands) are considered. Despite the great diversity of glandular trichomes occurring in the Leguminosae (e.g., Solereder 1908; Metcalfe and Chalk 1950; Lackey 1978; Leelavathi and Ramayya 1983; Marquiafável et al. 2009; Matos and Paiva 2012; Marinho et al. 2015; Vargas et al. 2015; Vargas et al. in press) studies regarding the secretion processes and histochemistry of the secretory structures in legumes are rare.

The relationship between the histochemistry and subcellular organization of these trichomes helps us to better understand the probable functions of these glands in Cajaninae. Terpenoids, phenolic compounds (flavonoids and tannins), and alkaloids are examples of secondary metabolites present in the secretory structures of legumes (Wink 2003; Roshchina 2012). Of these groups of compounds, we observed terpenoids (essential oils) and phenolics in Cajaninae.

Among the main factors that influence lipidic and phenolic metabolism are soil moisture, light, and temperature; these abiotic factors may induce changes in the nature and quantity of the metabolites produced (Gouinguené and Turlings 2002; Ramakrishna and Ravishankar 2011; Sampaio et al. 2011; Singer et al. 2016). Species of the Cajaninae have diversified through "cerrado" and savanna environments (Grear 1970, 1978; Schrire 2005), where there is high light incidence, high temperatures, and low availability of ground water (Furley and Ratter 1988). Most likely, the lipids (essential oils and total lipids) and phenolic compounds produced by the glandular trichomes of the Cajaninae help this group of plants to survive in these environments because, as suggest by Gouinguené and Turlings (2002), plants growing in dry environments invest more in the synthesis of defense compounds. According to these authors, when water is available, plants can invest more in growth and this mitigates against herbivory, whereas when less water is available, growth is reduced and protection of the vegetative parts becomes more important.

Phenolic compounds may act against a variety of herbivores and pathogenic microorganisms (Mazid et al. 2011), as well as various kinds of abiotic stresses (Feucht et al. 1994), including the absorption of ultraviolet light (Harborne 1977). In a brief review of phenolic compounds secreted by plants, Castro and Demarco (2008 and citations therein) mention that glandular trichomes secreting these natural compounds are present in the Asteraceae, Cucurbitaceae, Lamiaceae, Oleaceae, Orobanchaceae, and Verbenaceae families, but not in Leguminosae. However, we observed that *R. minima* produces these metabolites in its bulbous-based trichomes and vesicular glands. Given that phenolics are associated with resistance to drought, the identification of these substances in the glands of the studied species suggests a clear ecological function in the Cajaninae, and this will be particularly important during predicted periods of increasing drought as a consequence of global warming.

Among the three types of secretory structures here studied, only the vesicular glands have a stalk cell. It appears that these stalk cells have a different function to the head cells. Stalk cells generally have very large vacuoles, in contrast to the head secretory cells which have dense cytoplasm (Werker 2000), exactly as occurs in vesicular glands of the Cajaninae. The stalk cell has a thick cuticle and cell walls. Similarly, the basal cell in the glandular trichomes observed in this study also has thick walls. We hypothesize that lipid

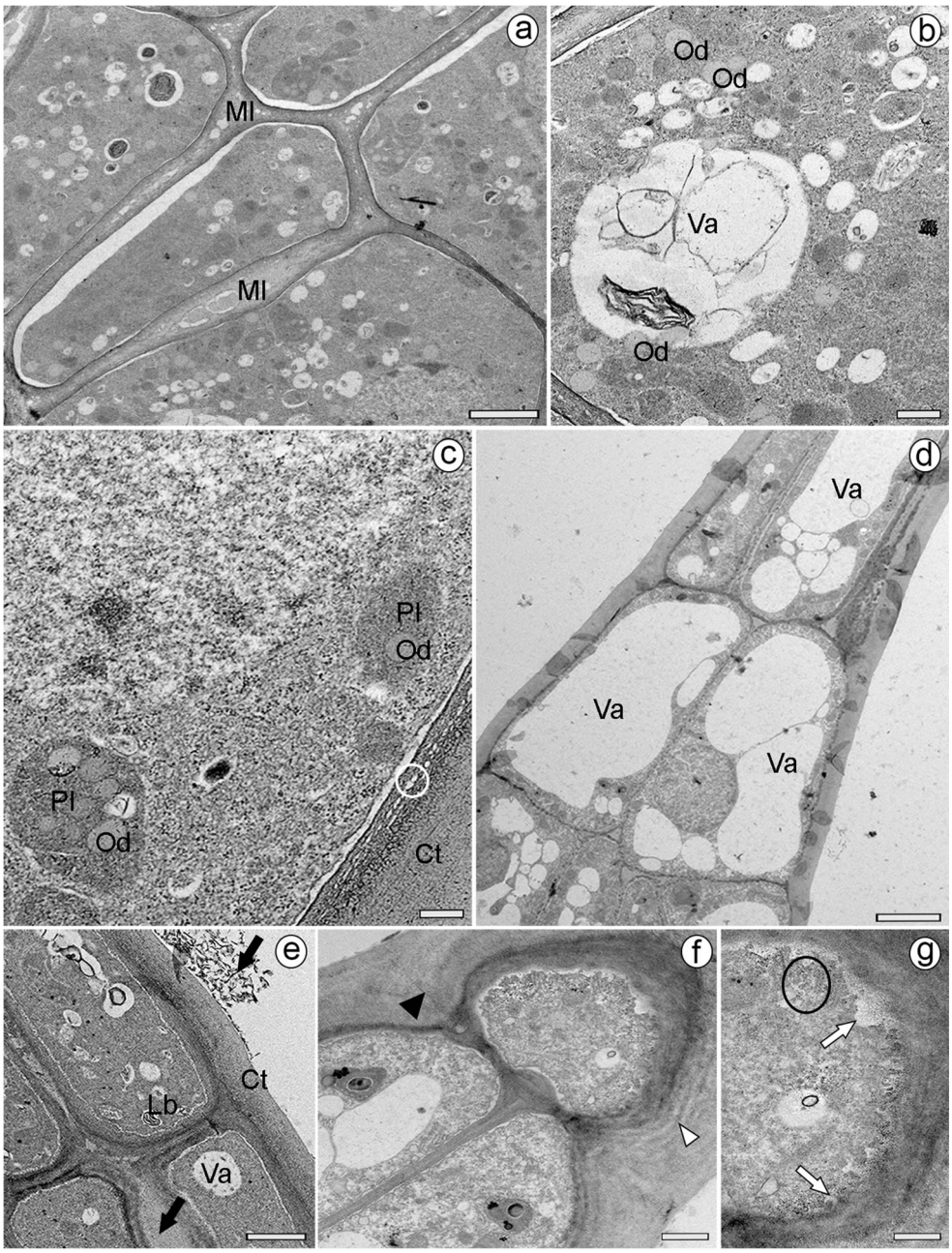


Fig. 5 Transmission electron micrographs (TEMs) of the bulbous-based trichomes in *Eriosema rufum* (**a**–**c**) and *Rhynchosia minima* (**d**–**g**). *Bulbous region* (**a**–**c**). **a** Progressive dissolution in the middle lamella forming large tubular space between inner cells. **b** Cells with oil drops scattered in the cytoplasm and in contact with the tonoplast. **c** Inner cells showing plastids with oil drops and cell walls presented an inner reticulate layer and outer fibrous layer (white circle), with fibrils protruding into the cuticle. *Neck region* (**d**–**g**). **d** Proximal cells with large vacuoles and reduced cytoplasm **e** Distal cell with abundant cytoplasm and few vacuoles exhibiting lamellar bodies. It is possible to observe that the secretion (black arrow) is accumulated in the periplasmic and intercellular space, cell walls, and on cuticle. **f** Terminal cell exhibiting poly-lamellate outer walls (white triangle), with evident transversal line of breakage (black triangle) between this cell and the underlying neck cells. **g** Labyrinthic walls (white arrow) in terminal cell and microtubules (black circle) arranged perpendicularly to the cell wall. Ct cuticle, Lb lamellar bodies, Ml middle lamella, Od oil drops, Pl plastids, Va vacuole. *Scale bars* **a**, **b**, **d**–**g** = 1 μm; **c** = 5 μm

incrustation in the lateral walls of the stalk and in basal cells forms an apoplastic barrier which regulates directional transport of metabolites to the glandular cells above (Werker 2000; Lange and Turner 2013; Sadala-Castilho et al. 2016). An additional function of the thick stalk walls and the basal cells is to support the head of the gland, as seen in glands of the plant family Velloziaceae (Sadala-Castilho et al. 2016).

Considering the organelles observed in glandular trichomes of the Cajaninae, the smooth endoplasmic reticulum and plastids devoid of thylakoids and with lipid inclusions are common characteristics which have a function in the biosynthesis of oil (Schnepf 1974; Fahn 1979, 2002; Lange and Turner 2013; Sá-Haiad et al. 2015; Tresmondi et al. 2017), as seen in the oil-secreting structures of other genera of the Leguminosae (Paiva and Machado 2007; Teixeira and Rocha 2009; Rodrigues et al. 2011; Matos and Paiva 2012; Rodrigues and Machado 2012). Lipid inclusions and osmiophilic material in cell walls, oil drops scattered in cytoplasm and on the inside of the plastids, vacuoles containing lipid inclusions, and multilamellar bodies were all observed in some cells of the glandular trichomes studied. The TEM results are consistent with our histochemical tests. All secretory structures here studied (in the cuticle, subcuticular spaces, lateral walls, central cavity, and protoplasm) reacted positively to Sudan IV and Nile blue tests indicating the presence of lipophilic substances (Figueiredo et al. 2007). This type of secretion previously has been reported for other glandular

trichomes in legume species (Matos and Paiva 2012; Marinho et al. 2015).

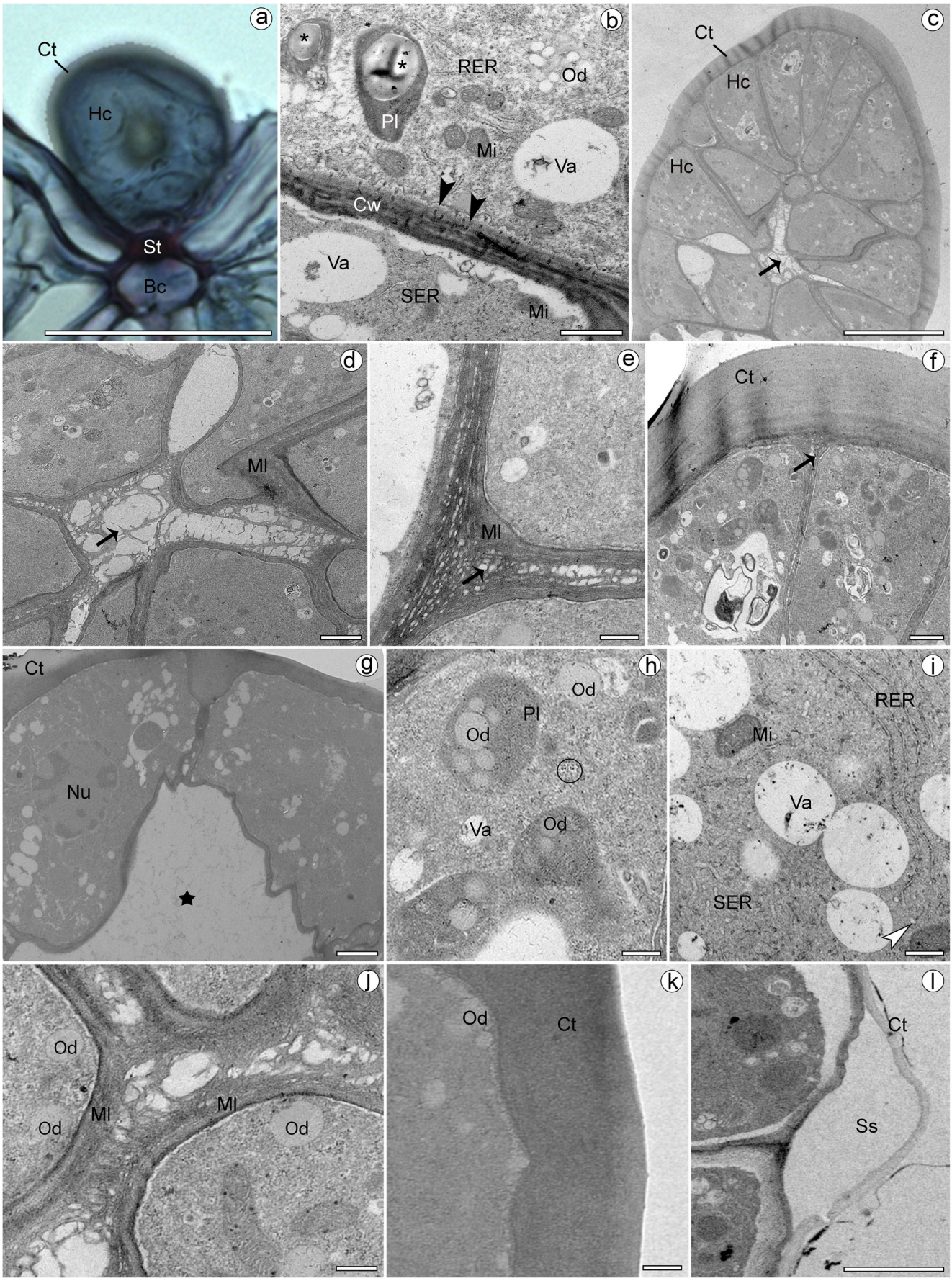
The occurrence of plastids with plastoglobules is another important feature of the cells in the glandular trichomes in the three studied species: *R minima*, *E. simplicifolium*, and *E. rufum*. Plastoglobules associated with thylakoids act as a reservoir of the lipids (Lichtenthaler 1968); plastoglobules also have a structural function, maintaining the shape of the lipid bodies and preventing their coalescence (Kessler and Vidi 2007). Additionally, a peripheral reticulum in the plastids was encountered in the basal cell (proximal) of the bulbous-based trichomes. The presence of thylakoids in these cells suggests that they have a photosynthetic function and are thus different from the cells of the other trichomes here studied. It is known that the peripheral reticulum increases the contact surface of the plastid with the cytoplasm and it appears to be involved in the movement of photosynthetic materials into and out of the plastid (Rosado-Alberio et al. 1968; Gracen et al. 1972). The peripheral reticulum probably functions in the transfer of the osmiophilic material to the chloroplast envelope, from where it passes to the endoplasmic reticulum and finally to the plasmalemma (Werker and Fahn 1981).

We also observed Golgi bodies in the head cells of the capitate trichomes. The presence of these organelles, in association with RER, indicates secretion of carbohydrates and proteins (Hawes 2005). We confirmed this with a positive reaction in the protoplasm of these cells using bromophenol blue histochemical tests. In addition, in all glandular trichomes in the Cajaninae species studied, there is a high density of ribosomes, polyribosomes, and rough endoplasmic reticulum, and these could be related to the intense cytoplasmic enzyme synthesis needed for cell metabolism (Turner and Croteau 2004). A hypothesis is that this intense cytoplasmic activity assists in the release of the gland exudates and the dissolution of the middle lamella, a process which occurs in all the glands here studied in subtribe Cajaninae and which is necessary for the cell wall gaps to develop (Machado et al. 2006).

Signs of granulocrine and eccrine mechanisms of elimination of the secretion from the protoplast were observed in the three types of glands studied here. Evidence of this includes the presence of scattered oil drops in the cytoplasm and near plasmalemma, multivesicular bodies, and numerous vesicles (scattered in the cytoplasm and near plasmalemma). The presence of oil drops near the plasmalemma facing the intercellular spaces suggests an eccrine secretion mechanism. In this secretion mechanism, the substance crosses the plasmalemma by active processes (Fahn 1979), then traverses the porous cell wall through the mechanical action of the protoplast with successive cycles of contraction and expansion, as suggested by Paiva (2016). On the other hand, the numerous coated vesicles and multivesicular bodies juxtaposed to the plasmalemma in the cells of the glandular trichomes of the Cajaninae indicate a granulocrine mechanism of secretion (Fahn 1979). In this process, the membrane of the vesicles fuses with the plasmalemma and the vesicle content is released from the protoplast (Fahn 1979). Images suggesting that the secretion reach the periplasmic space by exocytosis (granulocrine secretion) were frequently observed in most cells of the Cajaninae secretory trichomes. Alternatively, the vesicles are eliminated by invaginations of the plasmalemma (Fahn 1979), this probably occurring in the terminal cell in the neck region of the bulbous-based trichomes where we observed labyrinthic walls with microtubules arranged perpendicularly. The labyrinthic walls characterize transfer cells (Evert 2006), a common feature in salt glands, nectaries (Fahn 1979), and glandular trichomes (Owen Jr and Thomson 1991; Tozin and Rodrigues 2017). This structure enlarges the surface protoplast (Fahn 1979) and may facilitate release of the exudates (Gunning and Pate 1969).

Apparently, the cell walls in some cells of the Cajaninae secretory trichomes have variable electron density because the passage of material through them occurs. In addition, our results demonstrate the involvement of the secretory cell walls in the release of exudates, as discussed by Tresmondi et al. (2017). The loose aspect of these cell walls is a feature that aids the passage of exudates, because the loosely arranged cellulose microfibrils produce a porous cell wall that allows material from the cytoplasm to pass through them (Evert 2006). The formation of intercellular spaces through the displacement of the cells results from the dissolution of the middle lamella. This dissolution starts where more than two cells are joined in the head of a capitate trichome or vesicular gland, and in the bulbous region in bulbous-based trichomes. The accumulation of secretion in the intercellular spaces already has been reported for some glandular trichomes (Baucher and Holzl 1959; Akers et al. 1978; Possobom and Machado 2017). In the vesicular glands, these spaces are larger, and at maturity, it is possible to observe a wide space where the secretion is stored. This feature also was described for the peltate and cavitated trichomes of other Leguminosae taxa (Matos and Paiva 2012; Marinho et al. 2015). Furthermore, in the vesicular glands, we observed the detachment of the cuticle from the external cell walls, a feature previously noted in several types of glandular trichomes (Amelunxen 1964; Schnepf 1968; Cornara et al. 2001; Duke and Paul 1993; Matos and Paiva 2012). The secretion from the protoplasm of the cells of the gland head most probably ends up being stored in the subcuticular space, the origin of which seems to be related to the dissolution of the pectic layer between the outer periclinal cell wall and the cuticle, as described in other gland types (Possobom and Machado 2017).

In glandular trichomes, exudate release can occur via pores in the cuticle (Fahn 1979; Ascensão et al. 1999), by the rupture of the cuticle (Serrato-Valenti et al. 1997; Bisio et al. 1999), or even by diffusion through the cuticle (Serrato-Valenti et al. 1997). The release of exudates from the bulbous-based trichomes most likely occurs through the rupture of trichome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◀ **Fig. 6** Transversal section (**a**) and transmission electron micrographs (TEMs) (**b**–**l**) of the vesicular glands in leaflet of the *Eriosema simplicifolium*. **a** Vesicular gland with continuous cuticle, trapezoidal basal cell, a multicellular head with a central cavity, and inserted in an epidermal depression. **b** Detail of the basal and stalk cell: basal cell showing vacuoles with different size, mitochondria, and smooth endoplasmic reticulum (SER); stalk cell with few and small vacuoles, dense cytoplasm exhibiting mitochondria, rough endoplasmic reticulum (RER), plastids with starch grain (asterisk), and oil drop scattered in cytoplasm; thick tangential wall between basal and stalk cell with plasmodesmata (black arrowhead). **c** General view of head cells exhibiting central cavity developed by separation of adjoining primary cells walls through the middle lamella dissolution (black arrow). **d** Detail of middle lamella in dissolution (black arrow) characterizing schizogenous process in the head cells. **e** Detail of middle lamella in dissolution (black arrow) started in the corner of the inner head cell, advancing to other wall regions. **f** Middle lamella in dissolution (black arrow) reaching the cuticle in an apical head cell. **g** Broad intercellular space (black star) formed through of the displacement of the head cells towards the periphery of the vesicular gland. **h** Detail of head cell showing small vacuoles, polyribosomes (black circle), oil drops scattered in cytoplasm, rounded plastids devoid of thylakoids, and containing oil drops. **i** Detail of head cell showing mitochondria with well-developed cristae, extensive RER, proliferate SER, and numerous vesicles (white arrowhead). **j** Oil drop occurring in the peripheral cytoplasm near the plasmalemma facing the central cavity in head cells **k** Several oil drops occurring in the outer periclinal wall in the head cell **l** Formation of the subcuticular space in the region of the head of the gland. Bc basal cell, Ct cuticle, Cw cell wall, Hc head cells, Mi mitochondria, Ml middle lamella, Nu nucleus, Od oil drop, Pl plastids, RER rough endoplasmic reticulum, SER smooth endoplasmic reticulum, Ss subcuticular space, St stalk cell, Va vacuole. *Scale bars* **a** = 50 μm; **b**, **f** = 1 μm; **c**, **l** = 5 μm; **d**, **g**, **j** = 2 μm; **e**, **h**, **i**, **k** = 500 nm

cells by a line of breakage. A similar process also has been observed in some trichomes of the Asteraceae, where the breakage zone was described as a "line of weakness in the middle of the gland head" (Cornara et al. 2001), although the morphology of these glands in Asteraceae is completely different from the bulbous-based trichomes of the Cajaninae. In this peculiar glandular trichome of Cajaninae, the line of breakage is in the terminal portion of the neck region and the gland breakage and gland exudate release is readily caused by herbivory. In vesicular glands and capitate trichomes, exudates exit from the glandular head through the intact cuticle. The cuticle of the Cajaninae glandular trichomes becomes loosely organized but remains undamaged. We observed electron-dense bands in the cuticle; these bands probably are microchannels, as observed by Ascensão et al. (1997) in the reticulate cuticle of peltate trichomes in Lamiaceae species. Microchannels have also been reported in cell walls, as in *Hibiscus pernambucensis* Arruda (Malvaceae) (Rocha and Machado 2009); the authors noted that these structures increase the porosity of the wall to macromolecules. Ascensão et al. (1997) suggested that microchannels constitute the exit routes of the volatile substances of peltate trichomes of *Leonotis leonurus* (L.) R. Br.

The vesicular glands of Cajaninae morphologically resemble the cavitated glands of *Bauhinia* L., described by Marinho et al. (2015), with similar formation of a large intercellular space in the cells of the head of these two secretory structures by dissolution of the middle lamella. Nevertheless, the two gland types are different because vesicular glands have a rounded shape and a single-celled foot. The formation of the head cells occurs side by side, followed by dissolution of the middle lamella and then separation of the cells of the head. In the space formed by the dissolution of the lamella, there is deposition of the lipophilic content secreted by the cells that have moved away, and the exudate exits from the glandular head through the cuticle. In contrast, Marinho et al. (2015) observed a lenticular shape and biseriate foot in the cavitated glands of *Bauhinia*, and the internal space in the head is formed by the separation of cells from the apex towards the base, after which trichome elongation occurs only due to the increase in cell volume. Moreover, the secretion process in cavitated glands occurs through rupture of the cuticle.

Secondary metabolites are adaptive traits that have been subjected to selective pressures during evolution and are important for survival due to their defense function (Wink 2003). Our results suggest that the glandular trichomes in Cajaninae and their secretory compounds function as a defense against herbivores and help to prevent damage by UV radiation. The low incidence of herbivory (personal observation Vargas, W.) and the survival capacity in environments with a high light intensity and high temperatures of taxa in the Cajaninae appear to be strongly associated with the presence of glandular trichomes.

Despite the apparent functional and ecological importance of secretory trichomes in this plant group, a greater sampling of species is needed across the wide geographical distribution of the Cajaninae before the full functional importance of glandular trichomes can be understood.

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