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Anatomical structure and secretion compounds of colleters in nine *Ilex* species (Aquifoliaceae) from southern South America

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In order to clarify whether the structures observed at the base of the petiole of the genus *Ilex* are colleters resulting from stipules, the anatomy, vascularization and secretions of these supposed glandular structures were analysed in nine species. This is the first report of colleters in *Ilex*. Stipular colleters replace the stipules in all species studied and are characterized by the presence of vascular traces. In addition to the stipular colleters, three other types of colleter were distinguished: standard and lachrymiform colleters found on the leaf teeth or crenations, and sessile colleters found on the margins of the floral bracts. Their basic structure consists of a central core of parenchymatous cells surrounded by one layer of palisade secretory epidermal cells. Histochemical tests were also performed on secretions; proteins were found in the secretions studied, but glucose was not. The glandular origin of the stipular colleters is confirmed on the basis of their position, secretions and anatomy. Analyses of the colleter-secreted proteins distinguished two different groups of *Ilex* species. © 2009 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2009, **160**, 197–210.

ADDITIONAL KEYWORDS: anatomy – glandular – morphology – secretions – stipules – vascularization.

INTRODUCTION

The genus *Ilex* L., which belongs to the holly family Aquifoliaceae, is composed of deciduous and evergreen shrubs or trees with economic importance as crops (*Ilex paraguariensis* A.St.-Hil) or ornamentals (mostly temperate species). These plants are functionally dioecious; a rudimentary ovary (pistillode) is present in staminate flowers and sterile stamens (staminodes) in pistillate flowers. The genus contains more than 600 species, mainly distributed in America and Asia (Loizeau *et al.*, 2005; Loizeau *et al.*, in press). The main diversification areas of *Ilex* are South America and East Asia (Cuénoud *et al.*, 2000). Twelve species are present in southern South America, most growing in tropical or subtropical climates (Giberti, 1994, 1998, 2008). *Ilex paraguariensis* is the only species cultivated for industrial purposes; it is used to prepare a popular non-alcoholic beverage called 'mate', which is highly appreciated for its flavour and stimulating properties as a result of its caffeine and theobromine content (Filip *et al.*, 2001; Heck & de Mejia, 2007).

Ilex spp. may be recognized by their simple toothed or entire leaves with stipules that are minute to rudimentary, triangular-subulate, blackish, glabrous or pubescent. The inflorescences have bracts of similar form and size to the stipules (Giberti, 1979).

Successful vegetative propagation of *I. paraguariensis* has been achieved (Sansberro *et al.*, 1999; Tarragó *et al.*, 2005). During anatomical observations of micropropagation of this species, the presence of secretions and glandular characteristics was observed in stipules, vegetative nodes and mature leaves (A. M. Gonzalez and J. R. Tarragó, pers. observ.). To identify the origin of the secretions, anatomical studies of the micropropagated shoots were undertaken, which showed that the stipules have a glandular structure, suggesting that they might correspond to colleters.

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Colleters are external secretory structures, characterized by the production of a viscous substance that covers, protects and lubricates the bud and young leaves of the plant (Thomas & Dave, 1989a; Evert, 2007). The anatomical structure of colleters consists of a parenchymatic cellular axis surrounded by a layer of secretory palisade-like epidermal cells (Thomas, 1991; Da Cunha & Vieira, 1997). Such structures have been described on leaves, stems, flowers and other organs of more than 60 dicotyledonous families (Thomas, 1991). Baas (1973) studied the anatomy of Aquifoliaceae and related genera, but did not mention the occurrence of colleters or the glandular nature of the stipules. There are no records of colleters in *Ilex*, although anatomical studies of leaves, nodes, twigs and bark have been reported (Baas, 1975; Spegazzini, 2002). The main objectives of the present work were to analyse the anatomy, the compounds in any secretions and the presence or absence of vascular tissue in the stipules, teeth of the leaf lamina margin and floral bracts of nine southern South American species of *Ilex* (*I. argentina* Lillo, *I. brasiliensis* Loes., *I. brevicuspis* Reissek, *I. dumosa* Reissek, *I. integerrima* Reissek, *I. paraguariensis*, *I. pseudobuxus* Reissek, *I. taubertiana* Loes. and *I. theezans* Reissek) in order to clarify whether they are stipules or colleters.

MATERIAL AND METHODS

Botanical material

The list of *Ilex* taxa studied, together with their accession numbers, origin and the collectors, is presented in Table 1. Plant material was mostly obtained from the Estación Experimental INTA Cerro Azul (EEINTACA) Germplasm Bank (Misiones, Argentina), where *Ilex* spp. are grown in glasshouses. Vouchers were deposited in the herbarium of the Instituto de Botánica del Nordeste (CTES). The morphological analysis was performed on fresh material: buds, branches and inflorescences at different stages of development were collected from five individuals per species.

Light microscopy

For anatomical study, buds, leaves, young stems and inflorescences of the nine species (Table 1) were fixed in alcohol–formaldehyde–acetic acid (90 : 5 : 5, v/v/v) (FAA), dehydrated with 'deshidratante histológico BIOPUR' S.R.L., according to Gonzalez & Cristóbal (1997), and subsequently infiltrated with paraffin wax (Johansen, 1940). Transverse (TS) and longitudinal (LS) serial sections were cut at 10 μm with a rotary microtome. The sections were then stained with a safranin (C.I. 50240)–Astra blue combination (Luque,

Table 1. Colleter types in the nine studied species of *Ilex*

Species	Node	Leaf margin	Floral bract	Accession number and source/collector
I. argentina Lillo	st	s	se	Germplasm bank N° 111, cultivated, seeds from Argentina, Tucumán, Acheral – El Nogallar. Schinini & Dematteis 31585
I. brasiliensis Loes.	st	–	–	Germplasm bank N° 59, cultivated, seeds from Brazil, Paraná, Rio Branco do Sul. Schinini & Dematteis 31605
I. brevicuspis Reissek	st	–	–	Germplasm bank N° 81, cultivated, seeds from Argentina, Misiones, San Martín. Schinini & Dematteis 31610
I. dumosa Reissek	st	s	–	Germplasm bank N° 7, cultivated, seeds from Argentina, Misiones, Campo Viera. Schinini & Dematteis 31645
I. integerrima Reissek	st	lc	–	Germplasm bank N° 56, cultivated, seeds from Brazil, Paraná, Fijucas do Sul. Schinini & Dematteis 31612
I. paraguariensis A. St.–Hil.	st	lc	se	Germplasm bank N° 53, cultivated, seeds from Argentina, Misiones, Oberá. Schinini & Dematteis 31603
I. pseudobuxus Reissek	st	–	se	Germplasm bank N° 67, cultivated, seeds from Brazil, Paraná, Pontal do Sul. Schinini & Dematteis 31606
I. taubertiana Loes.	st	–	–	Germplasm bank N° 124, cultivated, seeds from Brazil, Rio Grande do Sul, San Francisco de Paula. Schinini & Dematteis 31589
I. theezans Reissek	st	–	–	Germplasm bank N° 71, cultivated, seeds from Brazil, Paraná, San Mateo do Sul. Schinini & Dematteis 31622

lc, lachrymiform colleter; s, standard colleter; se, sessile colleter; st, stipular colleter.

Sousa & Kraus, 1996) and photographed using a Leica DM LB2 light microscope. Observations and photographs with a Leica MZ6 stereomicroscope were also carried out.

Scanning electron microscopy (SEM)

For observation under a scanning electron microscope, the buds and branches of the nine species, fixed in FAA, were dehydrated through a graded acetone series, critical point dried in CO_2, coated with gold–palladium using a Denton Vacuum Desk II sputter coater, and observed using a Jeol LV 5800 scanning electron microscope.

Biochemical analysis of secretions

Young apices with different types of colleter of *Ilex* spp. were washed with an extraction buffer (0.1 M Tris-HCl plus 0.1% Triton X-100, pH 8.0) and the secretions were filtered through 0.45-mm Millipore filters. The sample consisted of the secretions of all the colleter types present in each species. *Ilex argentina* was not included in the biochemical studies because we did not have fresh samples. Protein determinations were performed by the Bradford (1976) method using bovine serum albumin as a standard. The absorbance at 595 nm was measured on a UV–visible spectrophotometer. Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with the filtrate as described by Laemmli (1970). 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) were used as molecular mass standards for SDS-PAGE. For electrophoresis, a 10% acrylamide gel was used.

Cluster analysis was used to determine the similarity between protein profiles of colleter secretions of different species of *Ilex*. The data consisted of 14 characters (protein bands) scored for each of nine operational taxonomic units (OTUs). Each character was scored for the presence (1) or absence (0). The resulting OTU × OTU matrix served as the input in the calculation of a phenogram by the unweighted pair-group method using arithmetic averages (UPGMA). The Jaccard similarity coefficient was applied to construct a similarity matrix. Distortion of the phenogram was measured by computing the cophenetic correlation coefficient (r). NTSYS-pc software package version 2.1 (Rohlf, 1994) was used for the analysis.

Glucose content was assayed in colleter secretions of *I. paraguariensis* and *I. dumosa* by the glucose oxidase–peroxidase (GOD–POD) enzymatic method ('Glicemia enzimatica AA' kit for glucose determination; Wiener Laboratorios S.A.I.C, Argentina).

RESULTS

Details of the species studied and the distribution and type of colleters found are presented in Table 1.

External morphology and distribution and location of colleters

The colleters observed are always solitary and located on young parts of the plant in three different places: (1) at the leaf base, in the nodal area of the union of the petiole with the stem, where they replace the stipules (Fig. 1A, 2G); (2) on the crenations or teeth of the leaves (Figs 1B, 3G); and (3) in the margins of the floral bract, at the union with the pedicel (Fig. 1D). Their form varied between conical (Fig. 4A, B), finger-like (Fig. 3G), tear-shaped (Figs 3I, 4D) and hemispherical (Fig. 1F).

Anatomy

All the colleters observed had a similar anatomical structure, consisting of a main body (central core) of parenchymatous cells ensheathed by radially elongated epidermal cells forming a dense, unilayered palisade (Fig. 2A, C, D, G–I). The parenchyma cells are longitudinally elongated without intercellular spaces (Fig. 2A, F). The cytoplasm of the parenchyma cells stained strongly with safranin (Fig. 2C). These cells had thin cellulose walls (Fig. 2I–K). The palisade epidermal cells were secretory in nature, with a relatively large nucleus, dense cytoplasm and a convex, smooth external wall (Fig. 2A, D–E). The colleters were covered by a thin cuticle (Fig. 1H–J). In young colleters, cuticle rupture or separation from the epidermal cells of the colleter was not observed.

Type of colleter

So far, four types of colleter may be distinguished in the genus *Ilex*, based on their shape and/or location in the plants, vascularization and presence or absence of a stalk.

Stipular colleters

These are characterized by a triangular to deltoid form and the presence of vascularization (Fig. 2). This colleter type is present in all nine species studied (Table 1). They are located on the node, at the base of the leaves (Fig. 1A, E). The margin is smooth (Figs 3C–F, 4A, B, E–I), and less frequently divided into three to five lobules (Fig. 3A). In transverse sections, the stipular colleters are triangular to square

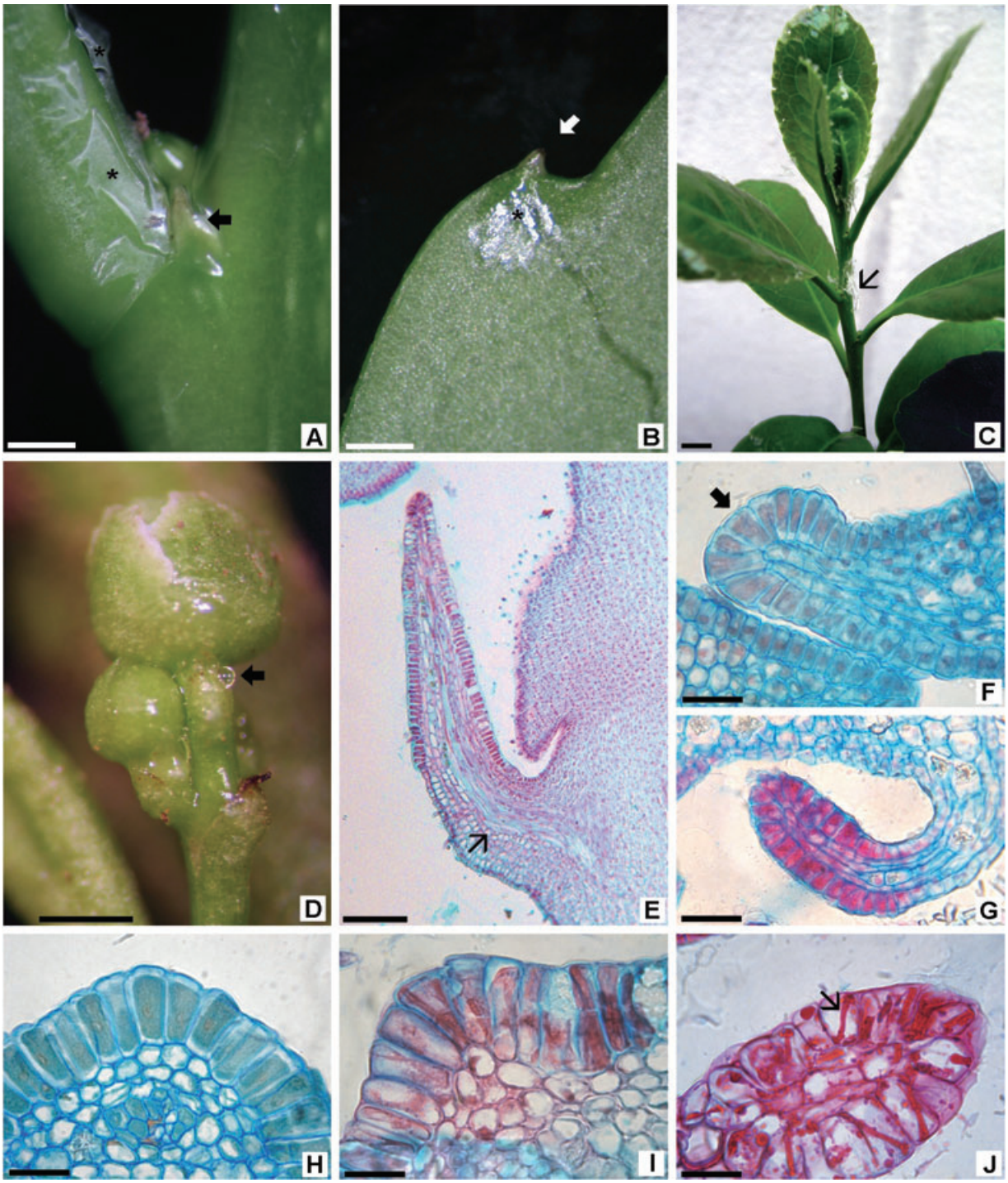

Figure 1. Colleter position, types and different stages of development. A–C, stereomicrographs of colleters. A, B, *Ilex paraguariensis*: A, leaf base showing a stipular colleter (arrow) and secretion (asterisk); B, leaf teeth with lachrymiform colleter (arrow) and secretion (asterisk). C, *I. taubertiana*, shoot apex with leaves and stipular colleters, secretion marked with thin arrow. D, *Ilex paraguariensis*, young inflorescence with a drop of secretion (arrow). E–J, light micrographs of colleters. E, F, *I. argentina*: E, longitudinal section (LS) of stipular colleter, the arrow indicates the vascular bundle; F, transverse section (TS) of floral bract shows a sessile colleter on the border (arrow). G, *I. dumosa*, TS of leaf tooth with standard colleter. H–J, *I. argentina*, details of colleters in three successive stages of development: H, TS of young stipular colleter; I, TS of mature stipular colleter; J, LS of standard colleter after ceasing secretory function; note the trabeculae in epidermal cells (arrow). Scale bars: A, B, D, 1 mm; C, 5 mm; E, 100 μm; F, G, 50 μm; H–J, 10 μm.

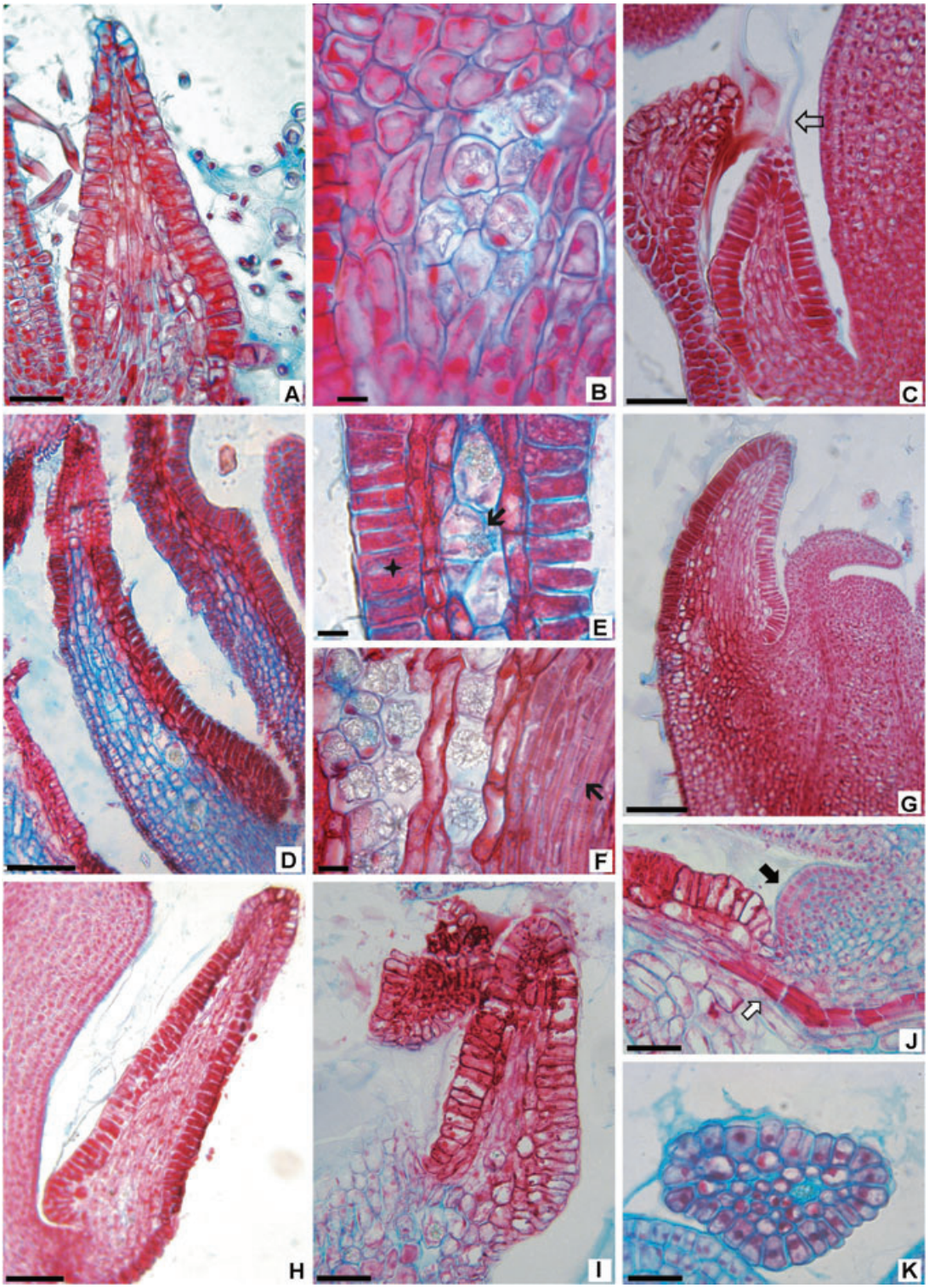

Figure 2. Light micrographs of stipular colleters. A, B, *Ilex brasiliensis*: A, longitudinal section (LS) of colleter; B, detail of Fig. 2A showing central core cells with nuclei and druses. C, *I. integerrima*, LS of colleter with secretion (arrow). D, E, *I. dumosa*: D, LS of colleter; note the basal constriction on the adaxial side; E, detail of Fig. 2D showing palisade epidermis (star) and elongated central cells with small druses (arrow). F, G, *I. pseudobuxus*: F, detail of Fig. 2G showing cells with druses and vascular bundle (arrow); G, LS of colleter. H, *I. theezans*, LS of colleter. I–K, *I. paraguariensis*: I, LS of colleter; J, detail of vascular bundle of colleter (white arrow) near the axillary bud (black arrow); K, TS of colleter. Scale bars: C, D, G, H, 100 μm; A, I, J, K, 50 μm; B, E, F, 10 μm.

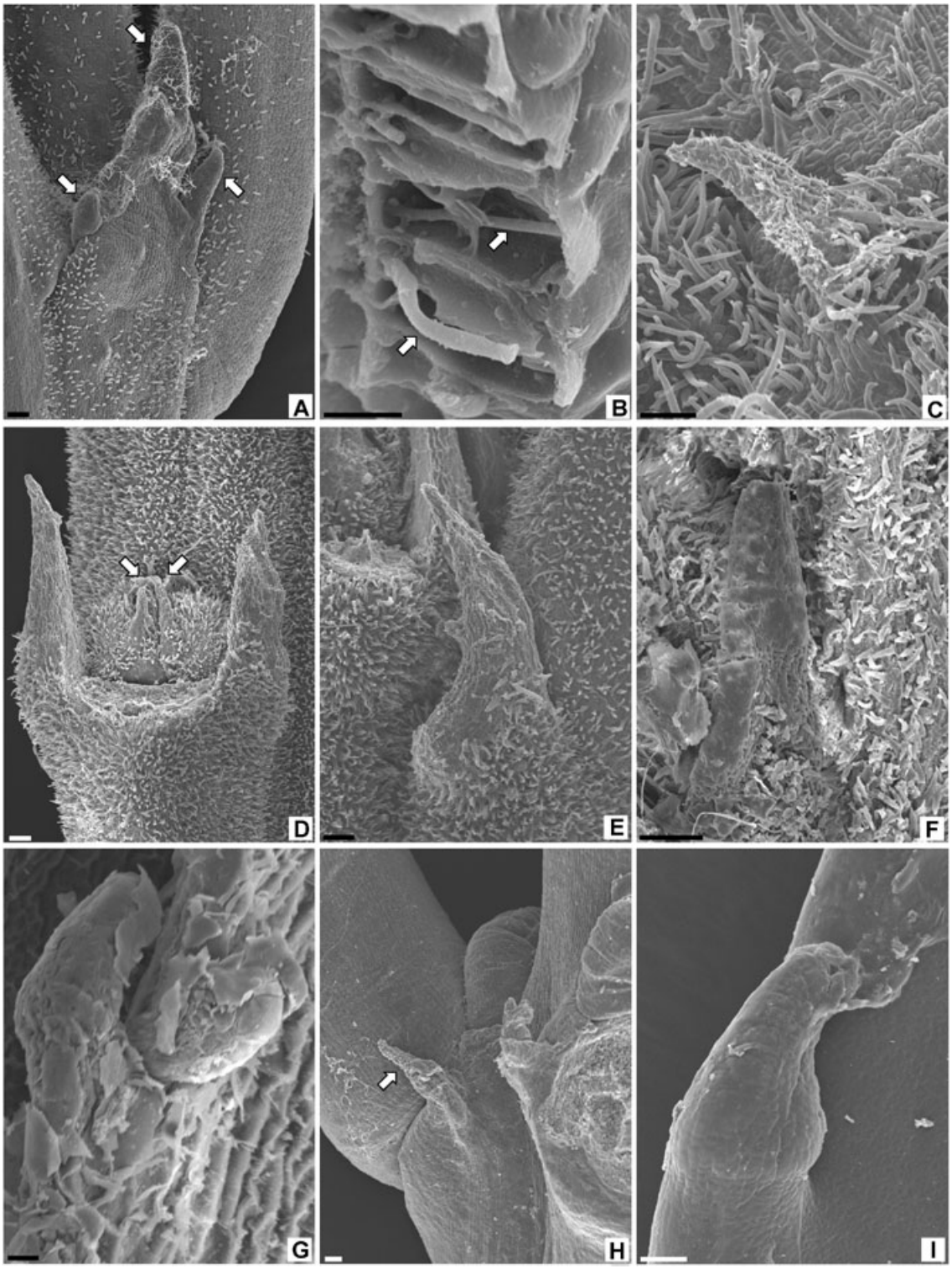

Figure 3. Scanning electron micrographs of colleters. A, B, *Ilex argentina*: A, node with stipular colleter, the lobules are marked with arrows; B, longitudinal section (LS) of stipular colleter showing trabeculae (arrows) in the palisade epidermis. C, *I. brasiliensis*, node with stipular colleter. D, E, *I. brevicuspis*: D, pair of stipular colleters after the fall of the leaf; note the axillary bud with young colleters (arrows); E, detail of colleter in Fig. 3D. F, G, *I. dumosa*: F, node with stipular colleter; G, teeth of leaf with standard colleter. H, I, *I. integerrima*: H, node with stipular colleters (arrow); I, teeth of the leaf with lachrymiform colleter; Scale bars: A, C–F, H, 100 μm; B, 10 μm; G, 25 μm; I, 50 μm.

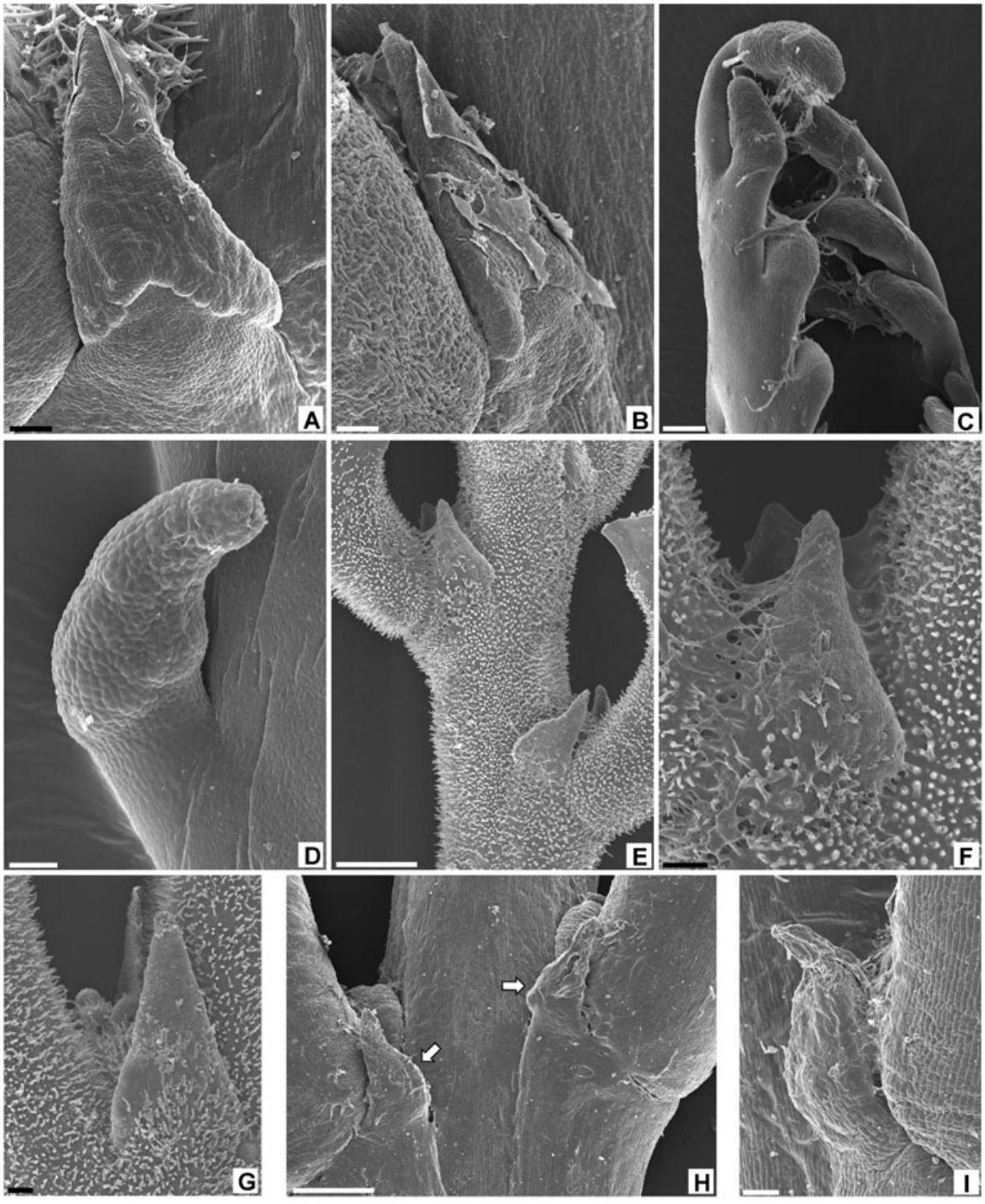

Figure 4. Scanning electron micrographs of colleters. A–D, *Ilex paraguariensis*: A, B, node with stipular colleter, without and with secretion, respectively; C, apical portion of leaf primordia showing lachrymiform colleters; D, detail of one lachrymiform colleter of leaf teeth. E, F, *I. pseudobuxus*: E, portion of young stem showing the position of stipular colleters in each node; F, detail of stipular colleter covered with secretion. G, *I. taubertiana*, node with stipular colleters. H, I, *I. theezans*: H, node with stipular colleters (arrows); I, detail of one colleter; Scale bars: A–C, F, G, D, 50 μm; E, H, 0.5 mm; I, 100 μm.

at the base and circular to elliptical at the apex (Fig. 2K). They have a basal narrowing, especially marked in the internal face (Fig. 2D, G, H–J). In some species, including *I. brevicuspis*, *I. dumosa*, *I. pseudobuxus* and *I. taubertiana*, they have simple unicellular trichomes in the basal and/or dorsal portion (Figs 3D–F, 4E–G); *I. brasiliensis* has pluricellular and uniseriate trichomes (Fig. 3C).

Anatomically, stipular colleters consist of an axis of elongated parenchyma cells with a few rows of 4–12 cells, which are crystalliferous, square and larger than the parenchyma cells from the main core of the colleter (Figs 1E, 2B, D–G). In young colleters, crystalliferous idioblasts retain their nuclei and are displaced laterally by the druse. During the senescence of the colleter, the crystal occupies the entire cellular lumen.

In some species, including *I. argentina* and *I. dumosa*, the palisade epidermis covers only the upper third portion of the abaxial face (Figs 1E, 2D). The cells of the adaxial face epidermis in *I. argentina* and *I. brasiliensis* are of similar length (Figs 1E, 2A). In *I. brevicuspis*, *I. integerrima*, *I. paraguariensis*, *I. pseudobuxus*, *I. taubertiana* and *I. theezans*, the basal cells are the longest (Fig. 2G, H, J); in *I. dumosa*, the basal epidermal cells are twice the length of the remaining epidermal cells, producing a bulge observable in a medial longitudinal section of the colleter (Fig. 4D). Except for *I. brasiliensis* (Figs 2A, 3C), all the studied species have a constriction at the base of the stipular colleters forming a stalk; in this zone, the parenchymatous core is covered by a non-glandular epidermis (Fig. 2H).

Standard colleters

These are present only in leaves of *I. argentina* and *I. dumosa* (Figs 1G, 3G). They are characteristically cylindrical, with a rounded apex and distinct stalk. They are glabrous and lack vascularization. The axis consists of parenchyma cells, without druses, covered by a single-layered palisade-like epidermis (Fig. 1G).

Lachrymiform colleters

These are present in leaves of *I. integerrima* and *I. paraguariensis* (Figs 3I, 4C–D), and differ in that the main body is tear-shaped or conical, with an acute and frequently curved apex (Figs 3I, 4C–D). They have a basal constriction or stalk, lack vascularization and are anatomically similar to the standard colleters. The standard and lachrymiform types are found on crenations or on the teeth of leaf primordia (Fig. 4C).

Sessile colleters

These are hemispherical and are found on the margins of floral bracts in *I. paraguariensis* and *I. pseudobuxus*. The axis is hemispherical or slightly conical, formed by 6–20 parenchyma cells. No druses or vascular bundles were observed in this type of colleter (Fig. 1F).

Secretions

The secretions of all types of young colleter are transparent, abundant, viscous when fresh (Fig. 1D) and cover the whole vegetative apex. The secretions are especially abundant in *I. paraguariensis*. The secretions form a white film when dry (Figs 1A, C, 4B, F). The colleters on the leaf teeth continue to produce secretions when the leaves have reached their mature size, although in smaller amounts (Figs 1B, asterisk; 3G). After ceasing their secretory function, the colleters gradually change their colour, from green to dark-brown. The stipular colleters persist as dry, wrinkled structures, even when the development of the leaf is complete.

At the beginning of senescence, the cytoplasm of epidermal cells becomes highly vacuolated, the nuclei disorganize and the cell wall becomes thickened (Fig. 1H). Later, the cytoplasm appears reddish with tanniniferous contents (Fig. 1I). Cellular necrosis begins in epidermal cells at the apex of the colleter and proceeds basipetally. Unusual structures, described as trabeculae, were observed in the secretory epidermis of *I. argentina* and *I. brasiliensis* during post-secretory stages. They appeared as bars or rods crossing the cell lumen, always diagonally from one external wall to the internal wall, the ends being slightly wider (Fig. 1J). Under SEM, they can be seen to leave a central furrow (Fig. 3B, arrow).

Vascularization

The standard, lachrymiform and sessile colleters lack vascularization (Fig. 1F–G), unlike the stipular type (Fig. 1E). All the studied species have trilacunar nodes with three traces (Fig. 5). In *I. paraguariensis* alone, the median leaf traces enter the petiole, whereas the lateral traces depart from the stele to the stipular colleters and then continue to take part in the petiole vascular supply (Fig. 5A–D). For the other studied species, like *I. argentina* (Fig. 5E–H), the median trace enters the petiole, and the stipular colleters are supplied by the lateral traces that do not take part in the petiole vascular supply. Each stipular colleter possesses one collateral vascular bundle, and the xylem is accompanied by parenchyma cells with druses (Fig. 2F).

Biochemistry of secretions

Proteins were detected by the Bradford method in the secretions of eight *Ilex* species assayed, with concen-

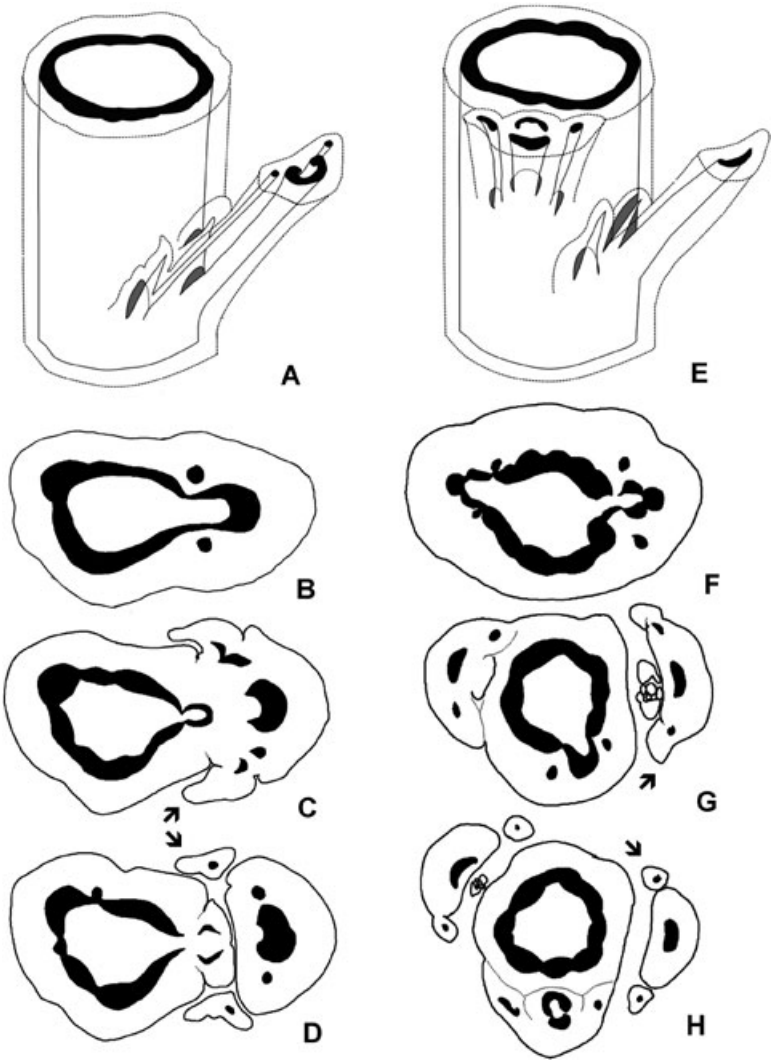

Figure 5. Schematic representation of the vascularization of stipular colleters: A–D, *Ilex paraguariensis*; E–H, *I. argentina*. A, E, reconstruction of nodal vascularization; B–D, F–H, transverse sections of the nodes at different levels; the arrows indicate the positions of the colleters.

trations ranging from 13 to 28 μg mL^{-1}. SDS-PAGE of *I. paraguariensis* (Fig. 6) showed that the secretions from colleters are composed of proteins. The molecular masses of the proteins, ranging from approximately 71 to 11 kDa, showed similar patterns between the different species (Fig. 7). Treatment with reducing agents performed on species with abundant secretions, *I. paraguariensis* and *I. dumosa*, showed no evidence of inter- or intra-chain disulphide linkages (data not shown). The similarity phenogram of characters for 14 OTUs discriminated two clusters with a cut-off limit of 0.5. One included *I. paraguariensis*, *I. dumosa*, *I. pseudobuxus* and *I. taubertiana* and the other *I. theezans*, *I. brevicuspis*, *I. brasiliensis* and *I. integerrima* (Fig. 8). Glucose was not present in the secretions of *I. paraguariensis* or *I. dumosa*.

DISCUSSION

This is the first report of colleters in *Ilex*. In order to determine whether the secretory structures present in the *Ilex* spp. analysed are colleters, stipules or other glandular structures, it was necessary to review the various definitions.

The term colleter was coined by Hanstein in 1848 (Foster, 1949) in reference to the sticky nature of the secretions. However, other terms have been attributed to these structures: glandular trichomes (Williams, Metcalf & Gust, 1982), resin glands (Curtis & Lersten, 1980), rudimentary stipules (Guedés, 1972; Rutishauser, 1999) and trichomes, emergences and glands (Fahn, 1990). A typical colleter consists of an emergence formed by an axis of parenchyma cells

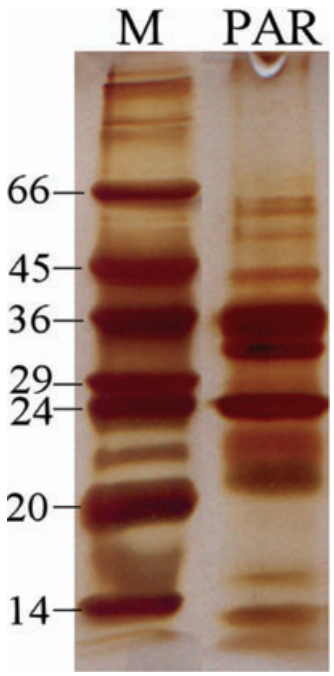

Figure 6. SDS-PAGE on 12.5% gel; protein detected by silver staining of *Ilex paraguariensis* (PAR) secretion. M, markers (kDa).

surrounded by a layer of palisade-like epidermal cells (Thomas, 1991; Da Cunha & Vieira, 1997; Evert 2007). The colleters can be located on the adaxial surface, the margin of the stipules (Lersten, 1974), the base of the leaf blade (Thomas, 1991) or the margins of leaf primordia or prophylls, or can replace stipules or prophylls (Gonzalez, 1998). The most distinguishing feature is the sticky secretion, a mucilaginous fluid that protects the meristem from desiccation, pathogens and herbivores (Dell, 1977; Thomas & Dave, 1989a, b; Thomas, Dave & Menon, 1989; Thomas, 1991). An additional characteristic is the early development of the colleters, which become fully developed when the leaves have not yet completed their expansion (Thomas, 1991). The different types of colleter in *Ilex* spp. are functional only during the initial development stages of the leaf, and senesce when the organ is totally expanded.

The glands or extrafloral nectaries are specialized tissues that secrete a sugary solution to reward animals. They can occur at the same sites as the stipules, and they can be sessile or stalked with or without vascularization (Elias, 1983; Koptur, 1992; Rico-Gray *et al.*, 2004; Nicolson, Nepi & Pacini, 2007).

Stipules are leaf-associated or basal appendages, typically located on both sides of the petiole base in the nodal region (Sinnott & Bailey, 1914; Howard, 1974; Dickison, 2000; Weberling, 2006). The presence of stipules is intimately associated with the vascularization of the nodal region (Sinnott, 1914; Sinnott & Bailey, 1914). These authors established that a correlation exists between lateral leaf traces and stipules. Unilacunar nodes are mostly exstipulate and trilacunar nodes are mostly stipulate. Since Sinnott's (1914) paper, *Ilex* has been cited as an example of a genus with trilacunar and unilacunar nodes (Baas, 1975). The species included in the present study have trilacunar nodes with three traces. Comparing the vascularization of the colleter, *I. paraguariensis* is the only species in which the traces that vascularize the stipular colleter branch off the lateral traces. The other species studied show a marked vascular reduction, as the lateral vascular traces diverge directly from the stem stele and supply the stipular colleter.

The secretions and the anatomical features observed in this study demonstrate that the structures located on both sides of the petiole base of *Ilex* are colleters. Furthermore, these structures originate from the abaxial surface of the leaf and do not develop marginal meristems like stipules. In contrast, the colleters in Rubieae (Rubiaceae) are accepted as rudimentary stipules (Rutishauser & Sattler, 1986; Rutishauser, 1999). In *Ilex*, the colleters are derived from lateral extensions from the leaf base, a position usually occupied by stipules, and produce abundant mucilaginous secretions.

Colleters in the stipular position or replacing the stipules have been reported in *Turnera* L. and *Piriqueta* Aubl. (Gonzalez, 1998), and in *Aphanopetalum* Endl. (Dickison *et al.*, 1994), and were described as minute stipules by previous authors. *Ilex laevigata* A.Gray is an example of this type, having foliage with two minute, gland-like basal stipules (Howard, 1974, 1979).

Stipular colleters are characterized by the presence of vascular traces. Vascularized colleters have been described in various species of Apocynaceae, for example, *Aganosma caryophyllata* G. Don. (Dave, Thomas & Kuriachen, 1987), *Mandevilla illustris* (Vell.) Woodson and *M. velutina* (Mart. ex Stadlem) Woodson (Appezzato-da-Glória & Estelita, 2000), and of Rubiaceae, for example, *Simira glaziovii* (K.Schum.) Steyerm (Klein *et al.*, 2004). According to Thomas (1991), vasculature in the colleter is connected to the organ to which it is attached; this feature was easily observed in the vascularization analysis reported here (i.e. in the stipular colleter of *I. paraguariensis*).

The colleters on the leaf teeth of *Ilex* are of standard or lachrymiform types according to the classifications proposed by Lersten (1974) for Rubiaceae and Gonzalez (1998) for Turneraceae. Sessile colleters in *Ilex* are restricted to floral bracts and appear to be a reduced form of the standard type, as in Turneraceae (Gonzalez, 1998).

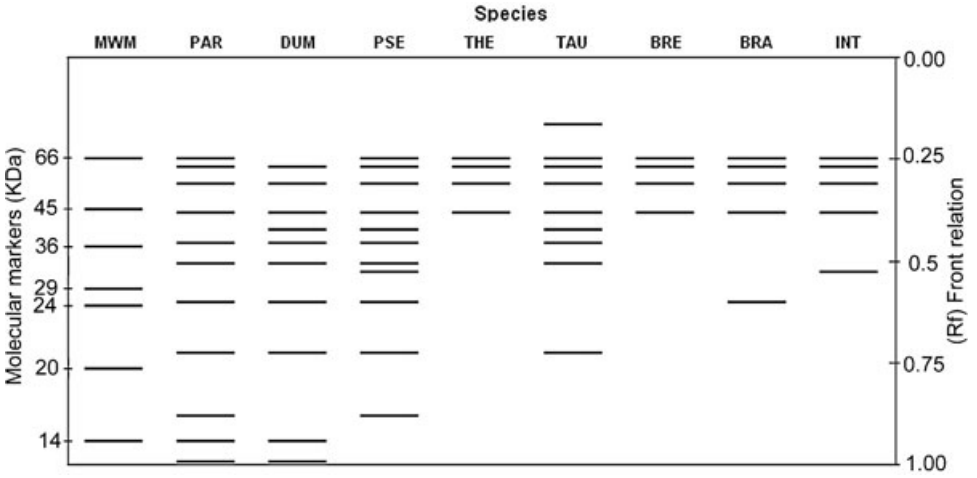

Figure 7. Schematic representation of the protein pattern of colleter secretion in *Ilex* species. BRA, *I. brasiliensis*; BRE, *I. brevicuspis*; DUM, *I. dumosa*; INT, *I. integerrima*; MWM, molecular weight marker; PAR, *I. paraguariensis*; PSE, *I. pseudobuxus*; TAU, *I. taubertiana*; THE, *I. theezans*.

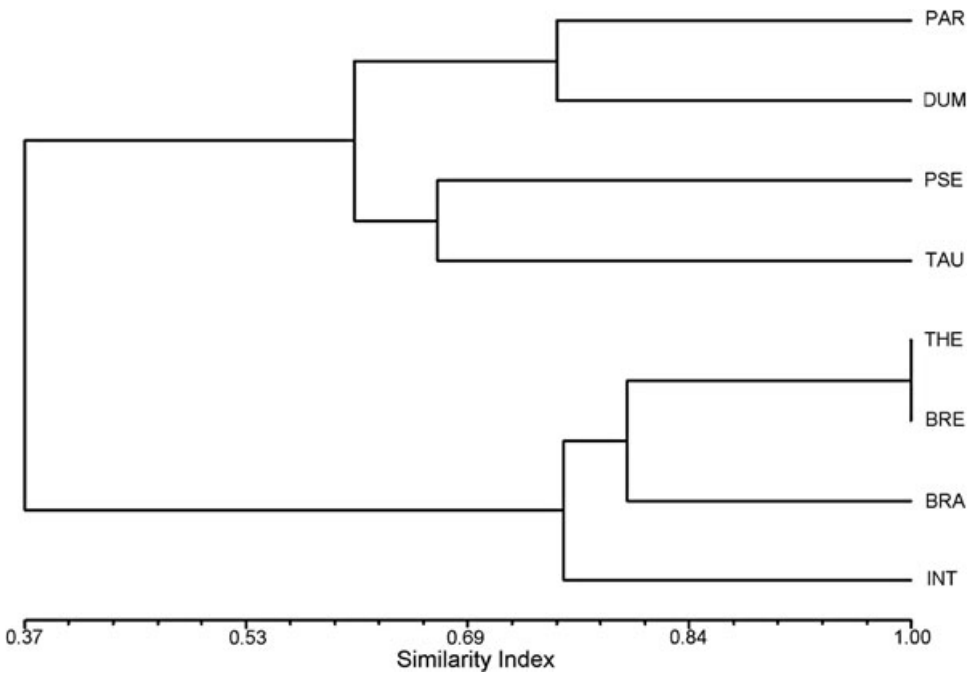

Figure 8. Phenograms of characters (protein bands) for 14 operational taxonomic units (OTUs) resulting from the unweighted pair-group method using arithmetic averages (UPGMA) cluster analysis of the OTU × OTU similarity matrix. Cophenetic correlation coefficient $(r) = 0.956$. BRA, *I. brasiliensis*; BRE, *I. brevicuspis*; DUM, *I. dumosa*; INT, *I. integerrima*; PAR, *I. paraguariensis*; PSE, *I. pseudobuxus*; TAU, *I. taubertiana*; THE, *I. theezans*.

The general anatomical structure of different types of *Ilex* colleter, with an axis and a palisade epidermis, is the same as described in other plants (Thomas, 1991; Gonzalez, 1998), and corresponds to the standard type described by Lersten (1974). Crystalliferous idioblasts are common in colleters of many angiosperm species and their position is often specific according to Thomas (1991). In *Ilex*, this is a general characteristic, found in the stipular colleters of all studied species. Another characteristic is the presence of a stalk at the base of the stipular colleters in *Ilex*. This character is poorly discussed in the literature, and was observed in a few species of Rubiaceae (Lersten, 1974; Da Cunha & Vieira, 1997; Klein *et al.*

2004). The presence of trabeculae in epidermal cells will require additional ultrastructural studies regarding the changes in the cytoplasm during colleter development.

The secretions start prior to the expansion of the leaf or the complete development of the inflorescence in all types of colleter in *Ilex*. Necrosis of the colleter begins at the apex, with the accumulation of dark contents in the cytoplasm and thickening of the epithelial cell walls. This degeneration proceeds from the apex to the base of the colleter, following the normal plan of development described in the literature (Curtis & Lersten, 1980; Durkee, Baird & Cohen, 1984). In lachrymiform or standard colleters of Turneraceae, the cuticle is distended, but no rupture or pore was observed and the secretion may be transcuticular (Gonzalez, 1998); a similar feature was seen in colleters of *Ilex*. According to Esau (1974) and Evert (2007), the colleters wither away when the leaves expand; this also occurs in stipular colleters in *Ilex*.

One of the main functions of the secretions of colleters is as a defensive mechanism against microorganisms and insects (Zalucki, Brower & Alonso, 2001; Cruz *et al.*, 2002). The presence of different protein exudates is related to meristematic tissue protection (Subroto *et al.*, 1996; Klein *et al.*, 2004). Our study demonstrates the presence of proteins in colleter secretions of *Ilex* spp., and this could be related to a protective or defensive function for the secretions, as mentioned by the authors cited above.

The species studied were arranged into two different groups based on the colleter secretion protein content. The clusters obtained from the patterns of protein bands in *Ilex* spp. show a probable specificity of the secretions among related species. These clusters were similar to the tree obtained by Gottlieb, Giberti & Poggio (2005) using amplified fragment length polymorphism.

Future studies will be conducted to elucidate the relationship between the presence of proteins in the secretions produced by colleters in southern South American *Ilex* spp. and their possible biological function against pathogens or insects and the protection of buds, especially in *I. paraguariensis*, which is an important crop in Argentina and Brazil.

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