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Anatomy of the extrafloral nectaries in species of *Chamaecrista* section *Absus* subsection *Baseophyllum* (Leguminosae, Caesalpinioideae)

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ABSTRACT

In this paper the ontogenesis and histochemistry of the petiolar glands found on the petiole/rachis of the eight *Chamaecrista* species of the section *Absus*, subsection *Baseophyllum* (Leguminosae, Caesalpinioideae) are studied by using light microscopy techniques, aiming to characterise these structures and to provide taxonomic characters which may be useful in phylogenetic approaches. Strips for glucose identification reacted positively with the exudates of the glands, confirming the presence of nectar in the secretion, characterising these glands as extrafloral nectaries (EFN). Histochemical tests also detected the presence of neutral and acid muco-polysaccharides, pectins, mucilages, total proteins, and phenolic compounds in the EFNs. The EFNs arise from a group of meristem cells (protodermis, ground meristem and procambium) in the petiole/rachis. All EFNs of the investigated taxa share some morpho-anatomical characters, so that their peculiarities are too weak to be used alone in the identification of particular species. Rather their similarities may be used to include these species into a single group, supporting the hypothesis of monophyly of the subsection *Baseophyllum*.

Introduction

Placed in the large family Leguminosae, *Chamaecrista* Moench includes about 330 species (Lewis, 2005) widely distributed in the tropical areas of the Americas, Africa and Asia, being most diverse in the New World (Lewis, 2005). According to Irwin and Barneby (1982), the genus is divided into six sections: *Apoucouita*, *Absus*, *Caliciopsis*, *Chamaecrista*, *Grimaldia*, and *Xerocalyx*. As a whole, sections *Absus* and *Grimaldia* possess sticky glandular hairs and lack extrafloral nectaries (EFNs), while the other four sections (*Apoucouita*, *Caliciopsis*, *Chamaecrista*, *Xerocalyx*) lack the sticky glandular hairs but are instead charged with EFNs.

Chamaecrista sect. *Absus* is further divided into four subsections: *Absus*, *Adenophyllum*, *Otophyllum* and *Baseophyllum*. In Brazil, the subsect. *Baseophyllum* is mainly distributed in the "campos rupestres" vegetation (rocky fields), sometimes also occurring in "cerrados" (Brazilian savanna), or separately in coastal "restingas" vegetation (sandy coastal plain), and in the "caatingas" vegetation (seasonally dry thorny forest): Irwin and Barneby (1978) and Conceição (2006).

The species sorted into sect. *Absus* subsect. *Baseophyllum* comprise a monophyletic group (Conceição et al., 2009) which display EFNs instead of typical sticky glandular hairs found in the species of the sect. *Absus*, an exception for the species of the sect. *Absus* (Conceição et al., 2008; Irwin and Barneby, 1982).

Nectaries are secretory structures that occur on the plant surface, being specialised for the secretion of a sweet solution called nectar (Elias, 1983; Nicolson and Thornburg, 2007; Roshchina and Roshchina, 1993). According to the topography, the nectaries have been referred to as floral nectaries, located on floral parts, and extrafloral nectaries (EFN), located on vegetative organs (Schmid, 1988).

Structures present on the petiole/rachis of the species included in the subsect. *Baseophyllum* were called by some authors "petiolar glands" (Irwin and Barneby, 1982) while others call them EFNs (Conceição et al., 2008, 2009). Information on the morpho-anatomical structure, composition of the exudates, and biological functions of these EFNs in species of the subsect. *Baseophyllum* and other species of *Chamaecrista* is scanty. Not even the carbohydrate nature of the exudates of the EFNs of *Chamaecrista trichopoda* (sect. *Chamaecrista*), could be confirmed by histochemical tests made by Francino et al. (2006), which emphasises the need for studies elucidating the real nature of the petiolar glands of *Chamaecrista* species. Additionally, other secretory structures secreting non-nectariferous substances have mistakenly been called EFNs due to their similar position and morphology to EFNs (Curtis and Lersten, 1978; Durkee et al., 1984).

Therefore, this paper aims (1) to provide morpho-anatomical information on the glands found on the petiole/rachis of the species belonging to the subsect. *Baseophyllum*; (2) to identify potentially

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useful taxonomic characters which may be used in phylogenetic approaches; (3) to histochemically identify the nature of the contents of the secretory cells, elucidating the kind of the glands which these species have. This could shed light on the conflicting terminology regarding the nature of the petiolar glands shown by the species which are included in the subsect. *Baseophyllum*. Finally, (4) the ontogenesis of these glands will be studied.

Materials and methods

The eight species of *Chamaecrista* are studied here, which are included in the subsect. *Baseophyllum*, as proposed by Conceição et al. (2008).

Samples of petioles and rachises of voucher material of the species belonging to the subsect. *Baseophyllum* were collected from two different herbaria (Table 1). These samples were rehydrated (Smith and Smith, 1942), dehydrated in an ethanol series, stored in 70% ethanol, and embedded in methacrylate resin (Historesin Leica, Leica Microsystems, Heidelberg, Germany). For structural characterisation, the blocks were cut with an automatic rotary microtome using glass knives (Leica RM2155, Deerfield, IL, USA) to produce 4-μm-thick sections. Sections were stained with toluidine blue at pH 4.0 (O'Brien and McCully, 1981) and the slides were mounted in resin (Permount, Fisher Scientific, NJ, USA).

Collections of fresh samples were also made (Table 1). Field expeditions were performed in April, October and November 2010. Samples from fresh material were fixed in FAA (formaldehyde, acetic acid and 50% ethyl alcohol; 1:1:18, v/v) for 48 h and stored in 70% ethanol (Johansen, 1940). Shoot apices, usually having 4 leaf primordia, and leaves from first and second nodes from fresh material were used for studying the ontogeny of the glands. Two species of *Chamaecrista* were selected for the ontogenetic study, *C. cytisoides* and *C. brachystachya*.

Samples stored in 70% ethanol were dehydrated through a tert-butyl alcohol series, and embedded in histological paraffin (Histosec®, Merck, Germany) according to Johansen (1940). The blocks were sectioned using a rotary microtome (Spencer 820 American Optical Corporation, Buffalo, NY, USA) and disposable stainless steel blades, producing cross and longitudinal serial sections 7 μm thick. For the ontogenetic and structural characterisations, sections were deparaffinised with xylene, hydrated, stained with 1% safranin and 2% astra blue (Roeser, 1972), dehydrated through ethyl alcohol/xylene series and mounted in resin (Permount). Some sections were also used in histochemical tests (Table 2).

The species used in the histochemical tests are summarised in Table 2. When fresh material was available, sections of fresh samples were made using an LPC table microtome (Rolemberg and Bhering Comércio and Importação LTDA, Belo Horizonte, Brazil). Fixed samples embedded in histological paraffin, treated as described above, were also used in the histochemical tests listed in Table 2. Controls for all histochemical tests were simultaneously carried out according to the protocol prescriptions. The material fixed with ferrous sulphate in formalin was embedded, sectioned and deparaffinised with xylene as described above for the material embedded in histological paraffin, and mounted in resin immediately after de-paraffinisation. All other slides used in the histochemical tests were mounted in glycerine gelatine.

During field expeditions we recorded which leaves contained secreting glands. Samples of exudates of five secreting petiolar glands of *Chamaecrista blanchetii*, *C. brachystachya* and *C. decora* were randomly collected and blotted on a urine test strip (Alamar Tecno Científica Ltda., São Paulo, Brazil) for glucose identification under field conditions. Branches of these three species were brought to the laboratory and kept in a bucket with tap water. The bucket with branches was bagged with a transparent bag for two days to maximise the humidity around the branches, which we thought would increase the exudation of the glands.

Observations, image captures, and paper photographic documentation were performed with a light microscope (model AX70TRF, Olympus Optical, Tokyo, Japan) equipped with a U-Photo system and a digital camera (model Spot Insightcolour 3.2.0, Diagnostic Instruments Inc., New York, USA).

Results

Rounded or elliptical concave elevated glands similar in shape were observed in all the species studied (Fig. 1A and B). All the species might display a few atypical flat glands. On rare occasions a pair of glands may also be found next to each other.

Gland ontogenesis

Neither macroscopic nor microscopic significant differences were observed in the ontogenesis process of the two species studied.

Macroscopically three major aspects were observed following each other during the development of the glands. At first, a cushion-like structure could be recognised on the petiole/rachis of the leaves of the shoot apex of both species. A slight concavity was then observed in the centre of this cushion-like structure. At the end, mature rounded or elliptical glands with a central concavity were observed in leaves of the first node onwards.

Four stages were microscopically observed, the first, second and third stages found in the leaf primordia of the shoot apex, while the fourth in the first through third node leaves. In the first stage (Fig. 2A), a cushion-like area comprised of cells with dense cytoplasm and nuclei was detected. Cells in the ground meristem of the gland primordium display a polyhedral shape, and conspicuous nuclei and nucleoli. Sometimes more than one nucleolus was observed. A high rate of cell divisions in the ground meristem occurs during this stage, comprising anticlinal, periclinal, and diagonal divisions. Long cells stemming from the procambium reach the adjacent area immediately below the nectary primordium. Two accessory bundles going through the differentiation process are already present at this early stage (Fig. 2A). Cells belonging to the protodermis of the gland primordium are cubical or slightly columnar-shaped, showing conspicuous nuclei and nucleoli, as observed for the ground meristem cells (Fig. 2A).

At the second and third stages, protodermal cells become columnar shaped (Fig. 2B and C). Although division in the protodermis is primarily anticlinal, a few periclinal divisions were also observed (Fig. 2B), characterising areas where the protodermis became bilayered. The major difference between the second and third stages relies on the size of the gland primordia and number of cells. Therefore, the gland primordia at the third stage are larger and with a higher number of cells if compared to the gland primordia at the second stage. Cell divisions are still persistent throughout the whole structure. The gland primordia of some *Chamaecrista cytisoides* samples were no longer rounded, appearing instead flat and wrinkled (Fig. 2D). The two or more accessory bundles become more evident (Fig. 2D).

Glands at the third stage reveal both xylem and phloem as being well-differentiated. Layers of long cells, which later would differentiate into fibres, were observed around the vascular tissues of the rachis and the accessory bundles. Below the ground meristem of the gland primordia, sclereids starting to develop their typical secondary wall thickenings were observed. At this stage, the gland shows a deepened central area, as macroscopically observed.

Table 1
Samples collected from the herbaria of the Universidade Estadual de Feira de Santana (HUEFS) and Universidade de São Paulo (SPF), and populations of *Chamaecrista blanchetii*, *C. brachystachya*, *C. coriacea*, *C. cytisoides* and *C. decora* with their respective location used in this study. Acronyms for Brazilian States: ES – Espírito Santo; MG – Minas Gerais.

Conceição et al. (2008)	Herbaria	Fresh material		Vegetation type where the species are found
		Municipality	Location	
C. blanchetii	HUEFS 66.389 HUEFS 131.380 SPF 90.113	Guarapari – ES (Parque Estadual Paulo César Vinha)	20°35'7.8"S, 40°25'17.3"W	campo rupestre, cerrado, restinga
C. brachystachya	HUEFS 12.469 HUEFS 17.060 SPF 179.567	Diamantina – MG (7 km East from Diamantina)	18°10'54"S, 43°33'45.5"W	caatinga, campo rupestre, cerrado, restinga
C. confertiformis	HUEFS 65.042 HUEFS 65.038 SPF 179.572	–	–	campo rupestre
C. coriacea	HUEFS 112.739 SPF 79.573	Conceição do Mato Dentro – MG (Gurutubas-Costa Sena)	18°43'31"S, 43°37'24"W	campo rupestre
C. cytisoides	HUEFS 105.351 HUEFS 71.567 SPF 160.397	Santa Bárbara do Monte Verde – MG (Três Cruzes-Serra Negra)	21°58'0.3"S, 43°49'12.1"W	transition between campo rupestre and fog forest (cloud forest)
C. decora	HUEFS 75.078 SPF 42.876	Diamantina – MG (Biribiri)	18°08'53.2"S, 43°36'53.1"W	campo rupestre
C. depauperata	HUEFS 94.669 HUEFS 112.632 SPF 79.580	–	–	campo rupestre
C. unijuga	HUEFS 101.773 HUEFS 73.833	–	–	restinga

Table 2
Chamaecrista species and type of material used in the histochemical tests. Species studied: 1 = *C. blanchetii*; 2 = *C. brachystachya*; 3 = *C. coriacea*; 4 = *C. cytisoides*; 5 = *C. decora*.

Metabolic group		Histochemical test	Type of material	
			Fresh material	Paraffin embedded material
Lipids	Lipid compounds	Sudan IV in 70% ethanol (Pearse, 1980)	2, 4, 5	2, 4, 5
Proteins	Total proteins	Xylidine Pounceau (O'Brien and McCully, 1981)	2, 4, 5	1, 2, 4, 5
Phenolic compounds	General phenolic compounds	Fixation with ferrous sulphate in formalin (Johansen, 1940)	–	1, 2, 3, 4, 5
	Lignins	Phloroglucinol (Johansen, 1940)	2, 4, 5	–
Polysaccharides	Total polysaccharides	Periodic acid schiff (Maia, 1979)	–	1, 2, 3, 4, 5
	Acid muco-polysaccharides	Alcian blue (Pearse, 1980)	–	1, 2, 3, 4, 5
	Pectins	Ruthenium red (Johansen, 1940)	–	1, 2, 3, 4, 5
	Mucilages	Tannic acid/ferric chloride (Pizzolato and Lillie, 1973)	2, 4, 5	1, 2, 3, 4, 5
	Starch	Periodic acid schiff (Maia, 1979)		1, 2, 3, 4, 5

Well-developed glands showing rounded margins and a well-defined central concavity occur in the fourth stage, as observed in mature glands (Fig. 2E). Now, the wrinkled surfaces of the glands of *C. cytisoides* become rounded at the borders, and the central concavity also becomes evident. The difference between glands at the fourth stage and the mature glands is that secretion could not be observed within the secretory parenchyma of glands at fourth stage.

Finally, a vascularised mature gland, formed by a secretory parenchyma and a single-layered epidermis was observed.

Gland characterisation

All species included in the sect. *Absus* subsect. *Baseophyllum* contained a single-layered epidermis lacking stomata (Figs. 2E and 3A). Occasionally, two layers of cells were also observed in the

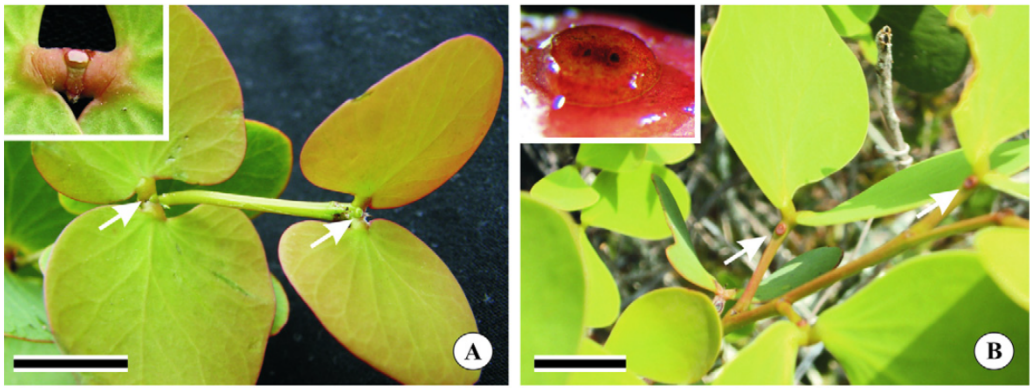

Fig. 1. (A) Extrafloral nectaries (EFN) (white arrows) of *Chamaecrista* species. (A) *C. blanchetii* showing the EFN found between the single pair of leaflets; (B) *C. decora* showing the EFN found on the petiole. In detail (A) and (B), EFNs covered with secretion. Scale bars = 2 cm.

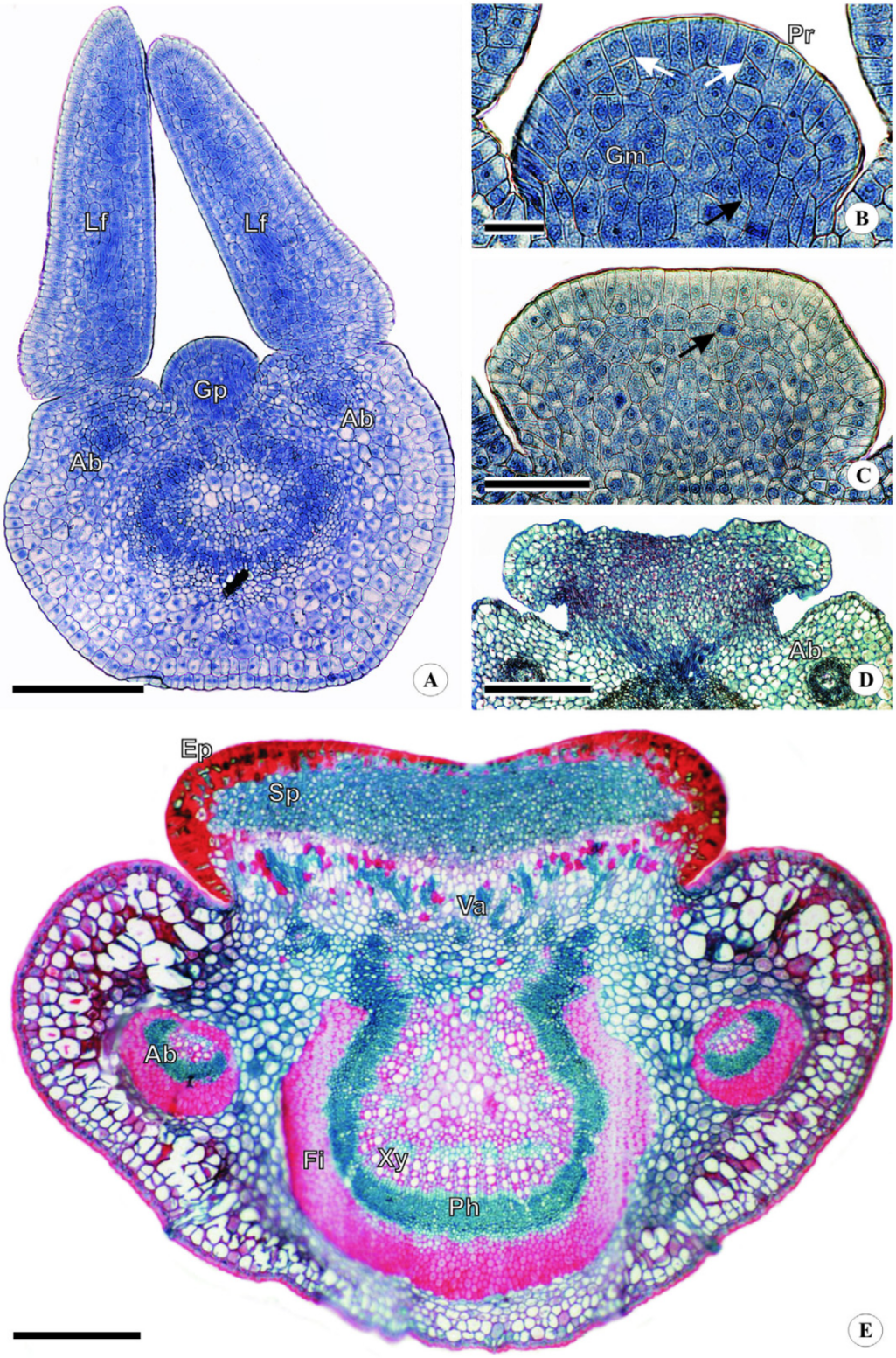

Fig. 2. Cross sections of the rachis showing the ontogenetic progress of the glands found in *Chamaecrista* species. (A–C, E) *C. brachystachya*. (D) *C. cytisoides*. (A) Gland primordium (Gp) at the first stage of development found between a pair of leaflet primordia (Lf). Note that the areas where accessory bundles (Ab) will be formed are already defined at this stage. (B) Gland primordium at the second stage showing the single-layered protoderm (Pr) and also areas where the protodermis is bi-layered (white arrow). Note the diving cells (black arrow) in the ground meristem (Gm). (C) Gland primordium at the third stage. (D) Gland primordium at the third stage showing wrinkled protodermis. (E) Mature extrafloral nectary gland (EFN). The EFNs are made up of a single-layered epidermis (Ep) which is subtended by a secretory parenchyma (Sp) supplied by vascular tissue (Va) abundant in phloem (Ph) and with little xylem (Xy) coming directly from the vascular system of the petiole/rachis. Two or more accessory bundles (Ab) are also found. Both accessory bundles and vascular tissue are surrounded by layers of fibres (Fi). Scale bars = 40 μm in (A) and (B); 100 μm in (C); 200 μm in (D) and (E).

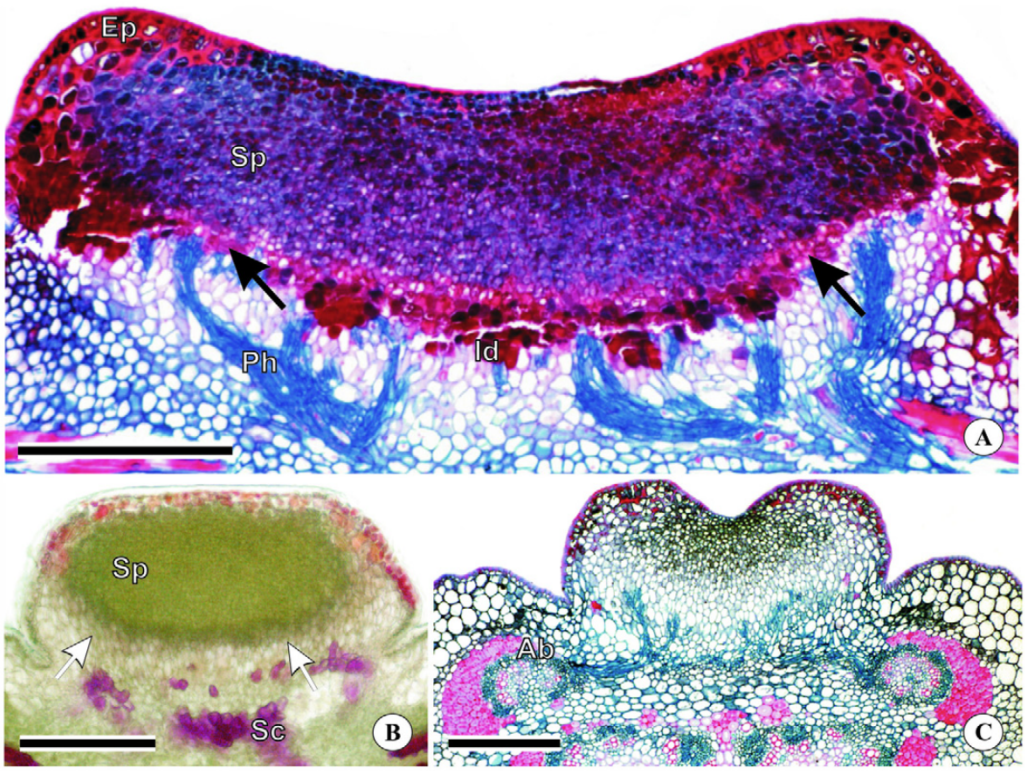

Fig. 3. Extrafloral nectaries (EFN) of *Chamaecrista* species in longitudinal (A) and cross (B and C) sections. (A) and (B) *C. decora* and (C) *C. coriacea*. (A) The EFNs are made up of a single-layered epidermis (Ep), secretory parenchyma (Sp), vascular tissue abundant in phloem (Ph). Note the presence of a layer of sclereids (black arrows) below the secretory parenchyma and clusters of mucilage idioblasts (Id) at the vascularisation endings. (B) Fresh sample section stained with phloroglucinol showing the transition zone (white arrow) and sclereids (Sc) found below the secretory parenchyma. (C) Note the two accessory bundles (Ab) found in the petiole converging towards the EFN. Scale bars = 300 μm.

epidermis. In general, at the centre of the gland where the concavity was found, the epidermal cells were cubical, thin-walled, and smaller than the cells at the margins of the glands (Figs. 2E and 3A). The epidermal cells at the margins were slightly columnar-shaped, showing thickened periclinal and anticlinal walls. Microchannels reaching the very top of the outer periclinal wall of the epidermal cells were observed through all the epidermal cells, being easily recognised at the margins of the glands, where the cells showed thicker walls.

The cells making up the secretory parenchyma were all polyhedral, with large nuclei and dark-staining cytoplasm (Figs. 2E and 3A), showing cellular spaces with secretions among each other. Hand sections of the glands revealed chloroplasts in the secretory parenchyma cells (Fig. 3B). The number of cell layers in the secretory parenchyma at the centre of the gland varied from 8 to 15 within and among species (Figs. 2E and 3A, B). Prismatic crystals were scantly and sparsely present in the secretory parenchyma. Vascular tissue did not enter the secretory parenchyma layers (Figs. 2E and 3A).

Immediately below the secretory parenchyma tissue, there was a transition zone between the secretory parenchyma and ground parenchyma cells (Fig. 3B). The transition zone was made up of two to three layers of polyhedral, lightly stained cytoplasm, and highly vacuolated parenchyma cells which displayed larger volume than the parenchyma cells from the secretory tissue. Idioblasts immersed in the transition zone and clusters of idioblasts (Fig. 3A) below the transition zone were also observed.

Glands were vascularised by xylem and phloem cells (Figs. 2E and 3A) coming directly from the vascular system of the petiole/rachis (Figs. 2E and 3A). However, the number of phloem cells outnumbered the amount of xylem cells. The accessory vascular bundles of some specimens of *Chamaecrista cytisoides*, *C. coriacea* and *C. decora* also vascularised the glands (Fig. 3C). Vascularisation going towards the glands, either from the petiole/rachis or accessory vascular bundles, ended among the cells of the parenchymatic zone right below the secretory tissue (Fig. 3A). Layers of fibres around the vascular tissue of the petiole varied from 5 to 10. When going toward the glands, the cells surrounding the xylem and phloem differentiated into sclereids (Fig. 3B) instead of fibres. The sclereids extended up until the parenchymatic zone right below the secretory tissue, forming a sheath of sclereids.

A sheath of idioblasts with prismatic crystals surrounding the layers of fibres around the accessory vascular bundles and the petiole/rachis vascular bundles was always observed.

Histochemical tests and glucose analysis

Our findings on the histochemical tests carried out for both fresh and paraffin-embedded material revealed the same results for all glands tested.

Total protein grains were detected in the secretory tissue of *C. blanchetii*, *C. brachystachya*, *C. decora* (Fig. 4A) and *C. cytisoides* (Fig. 4B).

General phenolic compounds were detected in epidermal cells, secretory tissue and within some cells in the transition zone of *C. blanchetii*, *C. brachystachya* (Fig. 4C), *C. cytisoides*, *C. coriacea* and *C. decora*. Phenolic compounds were observed as small or large dark drops which are filling the entire cytoplasm, forcing the nuclei to the cell periphery.

Lignins were detected in the walls of xylem cells, fibres and sclereids of *C. brachystachya*, *C. cytisoides*, *C. decora* (Fig. 3B).

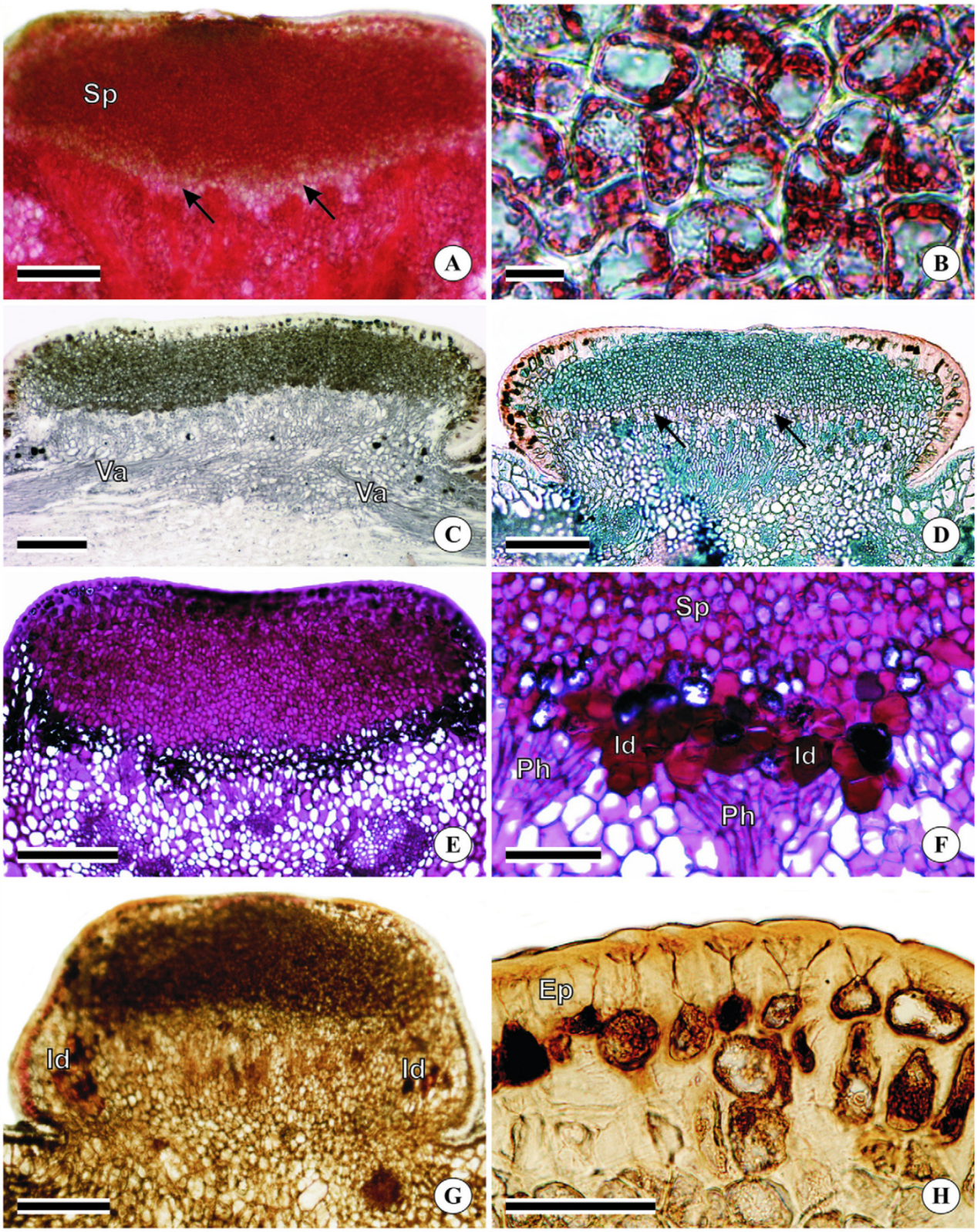

Fig. 4. Histochemical tests performed on cross sections of extrafloral nectaries (EFN) of *Chamaecrista* species. (A), (B), (G) and (H) Sections of fresh samples. (C–F) Paraffin embedded material. (A), (B) and (E–G) *C. decora*. (C–D) and (H) *C. brachystachya*. (A) and (B) Xylidine Ponceau. (A) Note the dark red staining of the secretory parenchyma (Sp) and that the transition zone (black arrow) is not stained. (B) Magnification of the secretory parenchyma of (A) showing the protein grains within the cells. (C) Fixation with ferrous sulphate in formalin showing the presence of general phenolic compounds. (D) Test with Alcian blue showing the presence of acid mucopolysaccharides staining. Note that the transition zone (black arrow) is not stained. (E) and (F) Periodic Acid Schiff. (F) Note the cluster of mucilage idioblasts (Id) found at the phloem (Ph) endings. (G) and (H) Tannic acid/ferric chloride showing the presence of mucilage in the (G) EFNs, mucilage idioblasts and (H) epidermis. Scale bars = 200 μm in (A), (C–E) and (G); 40 μm in (B), (F) and (H).

Acid muco-polysaccharides (Fig. 4D), neutral polysaccharides (Fig. 4E and F), pectins, and mucilages (Figs. 4G and H) were detected in the epidermal cells, in the secretory tissue, among the cells of the secretory tissue, and within some cells in the transition zone and the clusters of cells below the transitional zone (Fig. 4F). Neutral polysaccharides, pectins and mucilages (Fig. 4H) were clearly observed in the microchannels (Fig. 4H) of the epidermal cells of *C. blanchetii*, *C. brachystachya*, *C. cytisoides*, *C. coriacea* and *C. decora*. Acid muco-polysaccharides and mucilages rendered dubious results regarding their presence within the microchannels.

Starch was only detected in the ground parenchyma encircled by the vascular tissue of the petiole/rachis of *C. brachystachya*, *C. cytisoides*, *C. coriacea* and *C. decora*.

Strips for glucose identification did not react with the viscous exudates of *C. blanchetii*, *C. brachystachya* and *C. decora* in the field. However, when exudates were diluted with water and dropped onto strips for glucose identification, the strips then revealed the presence of glucose.

Secreting EFNs of young developing leaves and newly expanded leaves of *C. blanchetii*, *C. brachystachya*, *C. cytisoides* and *C. decora* did exude the most nectar under field conditions. Older leaves (i.e. from the seventh node on) were also observed to secrete nectar, but did so rarely and less often than younger leaves. Ants visiting the secretion of the glands were also observed but not collected for identification.

Discussion

Based on the position, external morphology, anatomy, detection of glucose in the secretion and polysaccharides, the petiole/rachis glands of the studied *Chamaecrista* species are characterised as extrafloral nectaries (EFN). Their development pattern (i.e. from the protodermis and underlying layers) is in accordance with other reports on such glands found in several plant families described in the literature (Coutinho et al., 2010; Paiva et al., 2007; Rocha et al., 2009; Thadeo et al., 2008).

At the third stage of development of some EFNs of *C. cytisoides*, the typical rounded area corresponding to the nectary primordium displays a wrinkled, non-rounded surface. That may be the result of asynchronous divisions in the nectary primordium. The protodermal cells probably undergo more divisions than the ground meristem cells, which cause the wrinkling of the gland surface. In addition, some of the protodermal cells also divide periclinally, which in turn can raise some areas in the protodermis leading to its wrinkling.

The EFNs present in *Chamaecrista* species of the subsect. *Baseophyllum* are made up of a single-layered epidermis which is subtended by a secretory parenchyma supplied by vascular tissue. According to Nepi (2007), nectaries may be anatomically differentiated into three areas: nectary epidermis, nectary parenchyma, and subnectary parenchyma. All three areas and the vascularisation supply abundant in phloem were observed in the EFNs of the *Chamaecrista* species belonging to the subsect. *Baseophyllum*. Similar structures have been reported in the literature in Leguminosae genera as well as in other families (Elias, 1983; Francino et al., 2006; Melo et al., 2010; Pascal et al., 2000).

A great difference on the EFNs described by other authors and the one we have studied is the presence of accessory bundles which may converge to the nectary gland. These accessory bundles may also be responsible for the supply of sugars used for nectar production.

Although the cells making up the single-layered epidermis were apparently non-secretory, our histochemical tests did find secretions within these cells. A single-layered epidermis made up of cubical cells was also observed in other species of Leguminosae (Elias, 1972; Pascal et al., 2000) and in *C. trichopoda* (Francino et al., 2006). This feature may be a common taxonomic anatomical character for the family Leguminosae.

As reported by several authors, the accumulation of secretion below a lifted cuticle (which later ruptures) is a general feature for EFNs (McDade and Turner, 1997; Nepi, 2007; Thadeo et al., 2008). However, as cuticular ruptures or detachments were not observed, we do not think that this is the case for the species studied here. Because the epidermis here was deprived of stomata and contained microchannels, nectar may be released through these microchannels as reported for other botanical families (Freitas et al., 2001; Koteyeva, 2005; Stpiczyńska et al., 2005; Weryszko-Chmielewska and Bożek, 2008). We are also compelled to believe that the main site of nectar exudation is the central area where thin-walled epidermal cells are found. The presence of thin-walled epidermal cells would weaken the barrier against nectar release. Moreover, Francino et al. (2006) reported the presence of cuticular pores at the centre of the EFN concavity which may be the sites for nectar exudation in *C. trichopoda.*

The presence of chloroplasts in the nectary parenchyma indicates that these plastids might be, along with the vascular tissue, directly contributing to the bulk of sugar secretion as suggested for some floral nectaries (Nepi, 2007; Pacini et al., 2003). Moreover, the lack of starch, which could be supplying the nectary parenchyma with sugar reserves, makes the presence of chloroplasts even more meaningful in the supply of energy for nectar production.

Presence of a subnectariferous parenchyma made up of thickened-wall cell layers subtending the secretory parenchyma which would act as a barrier to apoplastic transport, preventing secretion from reflux to the inner tissues, has been reported by other authors (Contreras and Lersten, 1984; Francino et al., 2006; Melo et al., 2010; Paiva et al., 2007). In the *Chamaecrista* species studied here, such wall thickenings were not observed. We considered that the subnectariferous parenchyma cells present in all EFNs of the *Chamaecrista* species studied represent a transition zone from the vascularised area to the non-vascularised area of the EFNs. Also, the lack of wall thickenings is important to guarantee a rapid flow of photoassimilates, nutrients and water from both the phloem and xylem sap. In addition, according to our histochemical findings, the idioblasts immersed in this subnectariferous parenchyma and the idioblasts forming clusters below this area store neutral polysaccharides, acid muco-polysaccharides, mucilage, and pectins. These substances might serve as an energy reservoir for nectar secretion as suggested by Fahn (1979). In fact, the mucilage idioblast cells in the *Chamaecrista* species studied here are specially located as clusters at the end of the vascularisation, which is mainly composed of phloem, corroborating the hypothesis that these mucilage idioblasts may act as a food reserve. Several authors have also proposed that the mucilage is involved in water storage (Fahn, 1979; Leitão et al., 2005; Sawidis, 1991).

Layers of fibres surrounding the vascular bundles are not present in the vascular tissue from the petiole/rachis or accessory bundles that converge towards the EFNs. Instead, sclereids were observed to form a sheath right below the subnectariferous parenchyma. Pascal et al. (2000) mentions the presence of a layer of sclerenchyma cells between the vascular tissue and the secretory cells of the EFNs of *Inga feuillei*, which could be homologous to the sclereid cells observed by us. Such sclereids may be acting in the mechanical support of the EFNs, as these EFNs are large and structured.

The presence of mucilage, pectins, proteins, and polysaccharides in the nectariferous parenchyma of EFNs suggests that such compounds might be found in the exudates, which is evidence for the nectar's complexity. The presence of such substances in EFNs has also been described by other authors (Caldwell and Gerhardt, 1986; Coutinho et al., 2010; Rocha et al., 2009). On the other hand, we think that the presence of phenolic compounds in the EFNs is

not necessarily an indication of its presence in the nectar. Phenolic compounds confer unpalatability and toxicity to the plant organs where they are produced, and which are then avoided by phytophagous and herbivorous animals (Nicolson and Thornburg, 2007; Roshchina and Roshchina, 1993). In a similar way, the presence of phenolic compounds in the EFNs of the species studied may confer unpalatability to these structures. They can also play a role ensuring immunity of the EFNs to bacterial or fungal infection (Roshchina and Roshchina, 1993).

In our study, the presence of total protein grains was found by using the dye xylidine Ponceau. These protein grains may be hydrolysed into amino acids which then become part of the exudates. Recent works have shown that ants tend to prefer solutions with amino acids over sugary solutions only (Lanza, 1991; Wagner and Kay, 2002; Wilder and Eubanks, 2009). Hence, one may assume that in the wild, ants would prefer nectars that are supplemented with amino acids. This behaviour suggests that plants with high levels of amino acids in their extrafloral nectars attract more ant protectors and therefore might suffer less herbivory than plants without amino acids.

As ants were observed visiting the EFNs, we believe that the role of the nectaries is related to ant/plant interactions. As observed for *C. debilis* (Nascimento and Del-Claro, 2010), it has long been shown that EFNs may be engaged in defensive strategies (Heil et al., 2000; Kost and Heil, 2005; McKey, 1989), which may help to increase the amount of fruit set (Oliveira et al., 1999). It is also relevant to highlight the correlation between the presence of the EFN in *Chamaecrista* sect. *Absus* subsect. *Baseophyllum* with the absence of sticky glandular trichomes, because if the secretion produced by the trichomes were present it would catch ants visiting the EFNs.

Under field conditions, EFNs of young developing leaves and newly expanded leaves of *C. blanchetii*, *C. brachystachya*, *C. cytisoides* and *C. decora* were observed to exude more nectar than older leaves. As a result, ants may be more attracted to the fragile young unfolding leaves that are more susceptible to herbivores. This concurs with what the optimal defence hypothesis predicts: the spatial allocation of defensive traits within a plant should favour more valuable and vulnerable plant areas (McKey, 1974). Young leaves are generally important for future plant fitness since they already have caused high construction costs without having contributed much yet to the plant's pool of photoassimilates (Radhika et al., 2008). Also, young leaves may lack other types of defences against herbivores, such as secondary metabolites, and effective mechanical tissues such as fibres (Harper, 1989).

Although the EFNs of *Chamaecrista* sect. *Absus* subsect. *Baseophyllum* do not present a nectary stalk, which is observed in several other *Chamaecrista* species, they do show morpho-anatomical similarities with the EFNs of species that present a nectary stalk, such as *C. trichopoda* (Francino et al., 2006). Such morpho-anatomical similarities among the EFNs present in several *Chamaecrista* species, included in different sections, suggest that the EFNs in genus *Chamaecrista* have a single origin, in agreement with the proposal of Conceição et al. (2009) who has considered the presence of EFNs in *Chamaecrista* a synapomorphy.

While the position of the EFNs on the petiole/rachis may aid the identification of some of the species included in the subsect. *Baseophyllum* (Conceição, 2006), their morpho-anatomical similarity limits their use in taxonomy. However, they can still have promising taxonomic value as they may be considered a conservative character, suggesting that the taxa included in the subsect. *Baseophyllum* are closely related. Moreover, as the histochemical tests detected the same metabolic compounds in the EFNs of all species studied, one may also consider such feature as a chemical conservative character that along with the morpho-anatomical similarities unifies the subsect. *Baseophyllum* as a monophyletic group, which may be used as an additional fact supporting the up-raking of subsect. *Baseophyllum* to sectional status suggested by Conceição et al. (2009).

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