ÍTALO ANTÔNIO COTTA COUTINHO

ANATOMIA FOLIAR COMO SUBSÍDIO PARA TAXONOMIA DE ESPÉCIES DE *CHAMAECRISTA* SEÇÃO *ABSUS* SUBSEÇÃO *BASEOPHYLLUM* (LEGUMINOSAE - CAESALPINIOIDEAE)

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Botânica, para obtenção do título de *Magister Scientiae*.

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ANATOMIA FOLIAR COMO SUBSÍDIO PARA TAXONOMIA DE ESPÉCIES DE *CHAMAECRISTA* SEÇÃO *ABSUS* SUBSEÇÃO *BASEOPHYLLUM* (LEGUMINOSAE-CAESALPINIOIDEAE)

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APROVADA: 22 de fevereiro de 2011.

Profª. Drª. Aristéa Alves Azevedo
(Co-orientadora)

Prof. Dr. João Marcos de Araújo

Profª. Drª. Renata Maria Strozi Alves Meira
(Orientadora)

Alguns confiam em carros e
outros em cavalos, mas nós
confiamos no nome do
Senhor nosso Deus.

Salmos 20: 6,7

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BIOGRAFIA

Ítalo Antônio Cotta Coutinho, filho de José Manoel Coutinho e Denise da Penha Cotta Coutinho, nasceu em Castelo, Espírito Santo, aos dias 7 de fevereiro de 1983.

Em abril de 2001 iniciou o curso de Ciências Biológicas na Universidade Federal de Viçosa, tornando-se bacharel em maio de 2006.

Em março de 2009, iniciou o curso de Mestrado em Botânica na Universidade Federal de Viçosa, concentrado seus estudos na área de Anatomia Vegetal.

SUMÁRIO

RESUMO

COUTINHO, Ítalo Antônio Cotta, M. Sc., Universidade Federal de Viçosa, fevereiro de 2011. **Anatomia foliar como subsídio para taxonomia de espécies de *Chamaecrista* seção *Absus* subseção *Baseophyllum* (Leguminosae - Caesalpinioideae)**. Orientadora: Renata Maria Strozi Alves Meira. Coorientadora: Aristéa Alves Azevedo.

Este trabalho tem por objetivo estudar as espécies de *Chamaecrista* circunscritas na seção *Absus* subseção *Baseophyllum*, fornecendo informações morfo-anatômicas da lâmina foliar, pecíolo/raque e glândulas encontradas no pecíolo/raque que possam ser úteis na separação das variedades circunscritas para *Chamaecrista cytisoides* (secção *Absus* subseção *Baseophyllum*) elevando-as ao nível de espécies, como definido por dados moleculares. A presença de glicose nos exsudados das glândulas presentes no pecíolo/raque confirmou que essas glândulas são de fato nectários extraflorais (NEF). Os NEFs são originados da protoderme, parênquima fundamental e procâmbio. Testes histoquímicos detectaram a presença de polissacarídeos neutros, muco-polissacarídeos ácidos, pectinas, mucilagens, proteínas totais e compostos fenólicos no tecido secretor dos NEFs, o que pode ser um indicativo da presença de tais substâncias no néctar. As oito espécies de *Chamaecrista* seção *Absus* subseção *Baseophyllum* apresentam epiderme unisseriada, venação do tipo actinódromo-camptódromo-broquidódromo, feixes vasculares colaterais em arco aberto, camadas de fibras e bainha cristalífera ao redor dos feixes vasculares, traqueídes alargados nas terminações das vênulas, NEFs, coléteres e tricomas tectores. Essas características foram consideradas como caracteres unificadores para esta subseção. A posição dos estômatos na folha, o tipo de mesofilo, o número de camadas tanto do parênquima paliçádico quanto lacunoso,

a presença de hipoderme, a posição dos idioblastos mucilaginosos nos folíolos e o número de feixes acessórios foram considerados caracteres úteis na distinção das espécies. A análise de componente principal (PCA) baseada e, caracteres morfo-anatômicos mostrou a separação clara entre as oito espécies incluídas na seção *Absus* subseção *Baseophyllum.*

ABSTRACT

COUTINHO, Ítalo Antônio Cotta, M.Sc., Universidade Federal de Viçosa, February 2011. **Leaf anatomy as an additional taxonomy tool for *Chamaecrista* species (section *Absus*, subsection *Baseophyllum* (Leguminosae - Caesalpinioideae)**. Advisor: Renata Maria Strozi Alves Meira. Co-advisor: Aristéa Alves Azevedo.

This paper aims at studying *Chamaecrista* species included in the sect. *Absus* subsect. *Baseophyllum* providing information on the morphology and anatomy of the leaflet blades, petiole/rachis, and glands found on the petiole/rachis which may be used on the separation of the varieties of *Chamaecrista cytisoides* (section *Absus* subsection *Baseophyllum*) into species, as defined by the molecular studies. The presence of glucose in the exudates of the glands confirmed that the petiolar/rachis glands are in fact EFNs. The EFNs arise from the protodermis, ground meristem and procambium. Histochemical tests detected the presence of neutral, acid muco-polysaccharides, pectins, mucilages, total proteins, and phenolic compounds in the EFN which may be indications of the presence of these substances in the nectar. All eight species of *Chamaecrista* sect. *Absus* subsect. *Baseophyllum* displayed a single-layered epidermis. Actinodromous-camptodromous-brochidodromous venation type, collateral vascular bundles arranged in open arc, layers of fibres and a sheath of cells containing prismatic crystals surrounding the vascular bundles, enlarged tracheids at the vein endings, vascular tissue of petiole/rachis with a pith parenchyma, accessory bundles, extrafloral nectaries, colleters, and non-secretory trichomes were observed in all the species studied and considered as unifying characters for this subsection. Position and type of stomata on leaves, type of mesophyll, number of both palisade and spongy parenchyma layers, presence of hypodermis, position of mucilage idioblasts in the leaflets, and number of accessory bundles were used to make

distinction among the species. Principal component analysis (PCA) based on morphological and anatomical characters showed the clear separation of the eight species included within sect. *Absus* subsect. *Baseophyllum*.

1. INTRODUÇÃO

O gênero *Chamaecrista* Moench está inserido na tribo Cassieae que é a segunda maior tribo da subfamília Caesalpinioideae, família Leguminosae (Irwin e Barneby 1982). Nesta família, inúmeras espécies são de reconhecida importância econômica, sendo largamente utilizadas na agricultura para produção de sementes destinadas à alimentação humana e a produção de forragens para animais, na indústria madeireira, na produção de fármacos, na extração de gomas, resinas e óleos, na fertilização do solo, na ornamentação e projetos paisagísticos, além de algumas serem plantas daninhas prejudiciais à agricultura (Atchison 1948; Joly 1966; Lewis *et al*. 2005).

A circunscrição dos gêneros incluídos na subtribo Cassiinae ainda é um assunto em discussão (Bruneau 2008). Atualmente, são reconhecidos três gêneros para Cassiinae: *Cassia, Senna e Chamaecrista* (Irwin e Barneby 1982; Lewis 2005).

A presença e a morfologia dos tricomas glandulares foram consideradas características úteis na taxonomia do gênero *Chamaecrista* (Irwin e Barneby 1982; Silva 1999; Tripathi e Sahu 1994). Nas seis seções definidas para o gênero, as estruturas secretoras se constituem importantes parâmetros taxonômicos. Nas espécies das seções *Absus* e *Grimaldia* os tricomas glandulares estão presentes, já nas seções *Apoucouita*, *Caliciopsis*, *Chamaecrista* e *Xerocalyx* estas estruturas estão ausentes, havendo a presença de tricomas tectores e predominância de nectários extraflorais (NEF) (Irwin e Barneby 1982). É interessante notar que NEFs ocorrerm em todas as epécies da subseção *Baseophylllum* que atualmente está incluida na seção *Absus*.

Francino (2006), em estudo comparativo da anatomia foliar de sete espécies de *Chamaecrista*, observou tricomas glandulares em duas espécies da seção *Absus* (*C. dentata* (Vogel) H.S.Irwin & Barneby e *C. hedysaroides* (Vogel) H.S.Irwin & Barneby). NEF's foram observados nas espécies da seção *Chamaecrista* (*C. mucronata* (Spreng.) H.S. Irwin & Barneby, *C. rotundata* (Vogel) H.S.Irwin & Barneby e *C. trichopoda* (Benth.) Britton & Rose ex Britton & Killip. Em *C. desvauxii* (Collad.) Killip, (seção *Xerocalyx*), embora tenham sido observados NEFs, estas estruturas apresentaram morfologia distinta das demais espécies, pois tinham tamanho reduzido, zona vascular pouco desenvolvida e o parênquima nectarífero pouco diferenciado. *C. rotundifolia* (Pers.) Greene (seção *Chamaecrista*) foi a única espécie que não apresentou estruturas secretoras.

Os estudos de morfologia e anatomia foliar são, reconhecidamente, ferramentas para a taxonomia e para a filogenia (Solereder 1908, Metcalfe e Chalk 1950, 1979, 1983). As características anatômicas, em especial das folhas, contribuem para elucidar problemas taxonômicos (Lersten e Curtis 1994; Moraes e Paoli 1999; Scatena *et al.* 1999; Kong 2001; Alves *et al.* 2002; Sartori e Tozzi 2002), além de permitir a identificação de amostras estéreis. Concomitantemente, a caracterização de estruturas secretoras pode ser crucial nas interpretações filogenéticas (Metcalfe e Chalk 1950; Bhattacharyya e Maheshwari 1971a, b).

Conforme abordagens filogenéticas, a presença de tricomas glandulares em folhas de *Chamaecrista* é considerada como uma condição mais derivada, e pode estar correlacionada com a ausência dos NEFs (Conceição 2006). Os resultados das análises filogenéticas indicam ainda que a subseção *Baseophyllum* deva ser elevada ao nível de seção, uma vez que esta constitui-se num grupo monofilético.

Conceição *et al*. 2008, em recente estudo biossistemático baseado em análises morfométricas e de aloenzimas , circunscreveu a subseção *Baseophyllum* reconhecendo oito espécies para o grupo: *Chamaecrista coriacea* (Benth.) H.S.Irwin & Barneby, *C. cytisoides* (Collad.) H.S.Irwin & Barneby, *C. depauperata* Conc., L.P.Queiroz & G.P.Lewis, *C. confertiformis* (H.S.Irwin & Barneby) Conc., L.P.Queiroz & G.P.Lewis, *C. brachystachya* (Benth.) Conc., L.P.Queiroz & G.P.Lewis, *C. decora* (H.S.Irwin & Barneby) Conc., L.P.Queiroz & G.P.Lewis, *C.unijuga* (Benth.) Conc., L.P.Queiroz & G.P.Lewis e *C. blanchetii* (Benth.) Conc., L.P.Queiroz & G.P.Lewis. Estas espécies apresentam nectários nas folhas e nas inflorescências, sendo que os tricomas glandulares estão ausentes.

Nectários são estruturas secretoras que podem ou não serem vascularizadas e ocorrem na superfície das plantas, sendo especializadas na secreção de néctar, uma solução açucarada composta principalmente por glicose, frutose e sacarose (Fahn 1979; Bentley e Elias 1983; Roshchina e Roshchina 1993). De acordo com sua posição podem ser classificados como florais (NF) ou extraflorais (NEF), quando presentes em peças florais ou em órgãos aéreos vegetativos respectivamente. A função dos NFs geralmente está relacionada à atração de agentes polinizadores (Fahn 1979; Bentley e Elias 1983; Nicolson 2007). Entretanto, o real papel biológico desempenhado pelos NEFs ainda é pauta de discussões em função da carência de trabalhos de campo.

Os NEFs foram inicialmente sugeridos como estruturas responsáveis pela secrecão de fotoassimilados produzidos em excesso (Mound 1962), não tendo necessariamente um benefício adaptativo. Entrentanto, não há um concenso em relação a tal hipótese, uma vez que um grande número de trabalhados e evidências

sugerem que os NEFs desempenham uma função protetora. O néctar produzido seria um recurso para os visitantes, especialmente formigas que apresentam um comportamento agressivo, auxiliando na proteção da planta contra a herbívoria (Roshchina e Roshchina 1993; Morellato e Oliveira 1994).

Outras estruturas também podem estar relacionadas com com a repulsão de agentes externos, como por exemplo tricomas que apresentam secreções de natureza lipofílica e/ou fenólica que atuando na defesa química contra o ataque de herbívoros e fitófagos (Werker 2000).

O gênero *Chamaecrista*, embora de distribuição neotropical (Lewis 2005), tem centro de diversidade no Brasil, na região do Planalto Central Brasileiro (Cerrado) e na Cadeia do Espinhaço (Campo Rupestre), onde ocorrem aproximadamente 250 espécies das 300 reconhecidas para o gênero (Conceição 2001; Irwin e Barneby 1978, 1982; Lewis 2005). O nível de desconhecimento sobre a biodiversidade brasileira ainda é muito grande. A crescente demanda por novos produtos químicos e especialmente fármacos vem aumentando cada vez mais o interesse sobre a biodiversidade existente no Brasil, principalmente a biodiversidade encontrada em áreas ainda pouco exploradas como os Campos Rupestres e nos biomas associados.

Além disso, a avaliação das estratégias adaptativas de espécies de *Chamaecrista* nas populações ocorrentes nos campos rupestres contribuirá para a compreensão de como os fatores ambientais podem influenciar no desenvolvimento das espécies, além de contribuir para o conhecimento da biodiversidade da flora desta formação vegetal. Este tipo de abordagem é crucial para o desenvolvimento de futuros trabalhos de manejo e bioprospecção.

2. OBJETIVOS

2. 1. Gerais

Os objetivos gerais da presente proposta são: descrever caracteres de valor taxonômico e filogenético que possam subsidiar a proposta de classificação apresentada por Conceição (2006) e Conceição *et al*. 2008, a qual eleva a subseção *Baseophyllum* ao nível de seção e delimita as oito espécies circunscritas para essa seção.

2. 2. Específicos

Os objetivos específicos deste trabalho são: (1) fornecer caracteres morfo-anatômicos dos folíolos e pecíolo/raque que possam ser úteis na separação das espécies encontradas na subseção *Baseophyllum*, como definido por estudos moleculares; (2) elaborar uma chave dicotômica baseada em dados anatômicos para separação destas espécies; (3) fornecer informações morfo-anatômicas das glândulas encontradas no pecíolo/raque das espécies pertencentes à subseção *Baseophyllum*; (4) identificar caracteres morfo-anatômicos que possam ser úteis na taxonomia deste grupo; (5) estudar a ontogênese das glândulas presentes no pecíolo/raque; e (6) identificar por meio da histoquímica a natureza do conteúdo presente nas células secretoras e proceder a análise da presença de glicose nos exsudados produzidos pelas glândulas, elucidando o tipo de glândulas presentes nessas espécies.

3. ORGANIZAÇÃO DA TESE

O presente trabalho encontra-se organizado sob a forma de artigos científicos, como disposto nas normas de redação de teses da Universidade Federal de Viçosa. Cada artigo segue a formatação da revista a que será submetido.

Os dois artigos se encontram nas normas da revista *Australian Journal of Botany*.

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Artigo 1

Anatomy of the glands found on the petiole and rachis of species of *Chamaecrista* section *Absus* subsection *Baseophyllum* (Leguminosae, Caesalpinioideae)

Anatomy of the glands found on the petiole and rachis of species of *Chamaecrista* section *Absus* subsection *Baseophyllum* (Leguminosae, Caesalpinioideae)

Ítalo Antônio Cotta Coutinho [1], Dayana Maria Teodoro Francino [1], Aristéa Alves Azevedo[1], Renata Maria Strozi Alves Meira[1]

[1]Federal University of Viçosa (UFV), Plant Biology Department, Viçosa 36.570-000, Minas Gerais, Brazil.

Corresponding author: Renata Maria Strozi Alves Meira (rmeira@ufv.br)

Abstract

This paper aims to provide taxonomic characters which may be used in phylogenetic approaches, as well as studying 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) by using light microscopy techniques. We also intend to provide ecological insights on plant/insect interactions to reveal the probable biological role played by these glands. Strips for glucose identification reacted positively with the exudates of the glands, confirming that these glands are in fact EFNs. The EFNs arise from a group of meristem cells (protodermis, ground meristem and procambium) in the petiole/rachis. Histochemical tests detected the presence of neutral and acid muco-polysaccharides, pectins, mucilages, total proteins, and phenolic compounds in the EFN, which may be an indication of the presence of these substances in the nectar. Although all EFNs share morphological and anatomical characters too weak to be used in the identification of particular species, such morphological and anatomical similarity still has promising taxonomic value as it can unify these species in a single group, giving support to the monophyletic hypothesis of the subsection *Baseophyllum*.

Key words: Ontogenesis, histochemistry, "campo rupestre", sandy coastal plain, savannah, subtribe Cassiinae, Brazil

Introduction

Placed in the large family Leguminosae, which consists of almost 730 genera and more than 19,300 species, *Chamaecrista* Moench includes about 330 species (Lewis *et al*. 2005) widely distributed in the tropical areas of the Americas, Africa and Asia, being the most diverse in the New World (Lewis 1987; Lewis *et al.* 2005).

Chamaecrista is placed within the subfamily Caesalpinioideae, tribe Cassieae, subtribe Cassiinae, which is the second largest subtribe of Cassieae (Lewis *et al*. 2005). Although the genus *Chamaecrista* had been established by Moench in 1794, it was only about 200 years later that the intense discussions regarding this genus came to fruition, when Irwin and Barneby (1981) proposed the separation of the genus *Cassia* into three distinct genera: *Cassia*, *Chamaecrista* and *Senna*, confirming *Chamaecrista* at the rank of genus (Irwin and Barneby 1982). The fine distinction between *Cassia*, *Chamaecrista* and *Senna* is also supported by recent molecular and phenetic data (Bruneau *et al.* 2001; Wojciechowski *et al.* 2004; Boonkerd *et al.* 2005; Bruneau *et al.* 2008; Laxmikanta *et al.* 2010).

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* display sticky glandular hairs and lack extrafloral nectaries (EFNs) on the petiole and/or leaf rachis, 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. One of the subsections of *Absus* is *Baseophyllum.* Although included within the section *Absus*, the subsect. *Baseophyllum* comprises a monophyletic group, bearing striking

differences in comparison to the other species belonging to the section *Absus*. The number of species and varieties listed in subsect. *Baseophyllum* has varied over time, depending on the author as well as different studies by the same author (Bentham 1870, 1871; Irwin and Barneby 1978, 1982; Conceição *et al*. 2008, 2009). The most widely used classification includes only two species in this subsection, *Chamaecrista cytisoides var. cytisoides* and *C. coriacea* (Irwin and Barneby 1982). However, molecular studies on the subsect. *Baseophyllum* (Conceição *et al*. 2008) support the recognition of eight species in this subsection.

The subsect. *Baseophyllum* is mainly distributed in the ‘‘campos rupestres’’ vegetation (rocky fields or rock outcrops), in the states of Bahia and Minas Gerais, Brazil, sometimes also occurring in ‘‘cerrados’’ (savannah-like vegetation), or separately in coastal ‘‘restingas’’ vegetation (sandy coastal plain) in the states of Pernambuco to Espírito Santo, Brazil, and in the ‘‘caatingas’’ vegetation (seasonally dry thorny forest) in the state of Pernambuco, Brazil (Irwin and Barneby 1978; Conceição 2006).

Contrarily to the other species included in the sect. *Absus*, EFNs are present in all species sorted into the subsect. *Baseophyllum*, while the typical sticky glandular hairs found in the species of the sect. *Absus* are lacking (Irwin and Barneby 1982; Conceição *et al*. 2008). In *Chamaecrista*, the presence/absence of EFNs and sticky glandular hairs were considered as useful taxonomical characters (Irwin and Barneby 1982; Conceição *et al*. 2008).

Nectaries are secretory structures that occur on the plant surface, being specialised for the secretion of a sweet solution called nectar (Fahn 1979a; Elias 1983; Roshchina and Roshchina 1993; Nicolson and Thornburg 2007).

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). On the other hand, based on the functional point of view, they have also been called nuptial nectaries when they are involved with the pollination system of the species, and extranuptial nectaries, when not directly associated with pollination (Schmid 1988).

While EFNs are common in the subfamily Mimosoideae, they are much less common in the subfamily Caesalpinioideae, being found mainly in the genera *Chamaecrista* and *Senna* (Lersten and Brubaker 1987; Pascal *et al.* 2000; Conceição *et al.* 2009). The occurrence, position, and anatomical structure of EFNs in *Cassia* are considered meaningful taxonomical characters for the genus (Bhattacharyya and Maheshwari 1971). However, such an approach has not been fully unravelled for *Chamaecrista*.

The structure present on the petiole/rachis of the species included in the subsect. *Baseophyllum* are called by some authors of petiolar glands (Irwin and Barneby 1982) while others call them EFNs (Conceição *et al.* 2008). Information on the anatomical structure, composition of the exudates, and biological functions of the so-called EFNs of the subsect. *Baseophyllum* is scanty. Not even the carbohydrate nature of the exudates of the EFNs of *Chamaecrista trichopoda* (sect. *Chamaecrista*), could be confirmed by the histochemical outcomes achieved by Francino *et al*. (2006), emphasising the importance of studies which elucidate 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 (Elias and Gelband 1977; Curtis and Lersten 1978; Durkee *et al.* 1984).

This paper aims (1) to give information on the morphology and anatomical characteristics of the glands found on the petiole/rachis of the species belonging to the subsect. *Baseophyllum*; (2) to identify potentially 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 glands bore by these species, shedding light on the conflicting terminology regarding the petiolar glands bore by the species included in the subsect. *Baseophyllum*; (4) to study the ontogenesis of these glands; (5) to provide ecological insights on plant/insect interactions, contributing to the understanding of the possible biological role played by these glands.

Material and Methods

Samples from petiole and rachis of voucher material of all the species belonging to the subsect. *Baseophyllum* were collected from two different herbaria (Table 1). Samples from voucher material were rehydrated by boiling in distilled water until completely submersed (Smith and Smith 1942), dehydrated in ethylic series, stored in 70% ethanol, and embedded in methacrylate resin (Historesin Leica, Leica Microsystems Nussloch Gmbh, Heidelberg) according to Meira and Martins (2003). 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).

Re-collection of fresh samples was also performed (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 apex, usually having 4 leaf primordia, and leaves from first and second nodes from fresh material were used for studying the ontogenetic process of the glands. As not all species were sprouting or had enough shoot apexes to perform the ontogenetic study, only two species were used in the ontogenetic study: *Chamaecrista cytisoides* var. *cytisoides* and *C. cytisoides* var. *brachystachya*.

Samples stored in 70% ethanol were dehydrated through a tert-butyl alcohol series, and embedded in histological paraffin (Histosec®, Merck, Germany) (Johansen 1940). The blocks were sectioned using a rotary microtome (Spencer 820 American Optical Corporation, Buffalo, NY, USA) and 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 as described in Table 2. Controls for all histochemical tests were simultaneously carried out according to the protocol requests. 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 deparaffinisation. 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 5 secreting petiolar glands of *C. cytisoides* var. *blanchetii*, *C. cytisoides* var. *brachystachya* and *C. cytisoides* var. *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). Glands will be classified according to Zimmermann's classification (1932) for nectary glands.

Results

Elevated glands varied regarding in shape and number on the leaves. The following characters were useful on the distinction of the species (Table 3). 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, *C. cytisoides* var. *brachystachya* and *C. cytisoides* var. *cysitoides*. Macroscopically, four stages could be observed. First, second and third stages were observed in the leaf primordia of the shoot apex, while the fourth stage was observed in the first through fourth node leaves.

In the first stage, a cushion-like area was observed on the rachis of both species. In the second stage, the cushion-like area became larger and more rounded. The surface of the gland primordia of *C. cytisoides* var. *cytisoides* was sometimes wrinkled at this stage. This was especially true for the glands found after the second pair of leaflets. In the third stage, the formation of a sunken central area giving rise to a slight concavity was observed. Most glands displayed the formation of the sunken central area. In the fourth stage, the enlargement of the gland and deepening of the central concavity were the only differences observed.

Four different development steps were microscopically observed. First, second and third steps were observed in the leaf primordia of the shoot apex, while fourth was observed in the first-fourth node leaves.

In the first stage, a cushion-like area, comprised of cells with cytoplasm and nuclei, darkly stained by astra blue and safranin, was detected in the cross section of the rachis. The ground meristem cells found in the gland primordium of the first

stage were smaller than the ground meristem cells belonging to other parts of the leaf primordium. Cells in the ground meristem of the gland primordium displayed a polyhedral shape, and conspicuous nuclei and nucleoli (Fig. 2*B–D*), with sometimes more than one nucleoli observed. A high rate of cell divisions in the ground meristem (Fig. 2*B–C*) was observed during this stage. Anticlinal, periclinal, and diagonal divisions occurred. Long cells coming from the procambium reaching the adjacent area immediately below this meristematic area were also observed. Two accessory bundles going through the differentiation process were already present at this early stage (Fig. 2*A*). Cells belonging to the protodermis of the gland primordium were cubical or slightly columnar shaped, showing conspicuous nuclei and nucleoli, as observed for the ground meristem cells (Fig. 2*A–C*). Although division in the protodermis was primarily anticlinal, a few periclinal divisions were also observed (Fig. 2*D, E*), characterising areas where the protodermis was bi-layered.

In glands observed in the second stage, cells belonging to the ground meristem were still smaller than the other surrounding ground meristem cells, while the protodermal cells became columnar shaped. However, cell divisions were still persistent throughout the whole structure. The gland primordia of some *C. cytisoides* var. *cytisoides* samples were no longer rounded, appearing instead flat and wrinkled (Fig. 2*E, F*). That was especially true for the glands found after the second pair of leaflets. The two accessory bundles become more evident (Fig. 2*F*), and although secondary wall thickenings are still light, xylem cells were easily recognised at this stage.

Glands at the third stage revealed both xylem and phloem (Fig. 2*G*) 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 (Fig. 2*H*). These cells showed narrower lumen than the other well-differentiated parenchyma cells. Below the ground meristem of the gland primordium, sclereids starting to develop their typical secondary wall thickenings were observed (Fig. 2*I*). The gland showed a deepened central area, as macroscopically observed. Although some undulations were still observed on the gland surface of some of the *C. cytisoides* var. *cytisoides* glands, the beginning of the formation of a deepened central area was also observed.

Well-developed glands showing rounded margins and a well-defined central concavity occurred in the fourth stage (Fig. 2*J*). The wrinkled surfaces of the glands of *C. cytisoides* var. *cytisoides* became rounded at the borders, and the central concavity also became evident (Fig. 2*J*). The walls of the sclereids and the cells forming the layers surrounding the vascular tissue in the rachis became thicker.

Finally, a vascularised mature gland, formed by a secretory parenchyma and a non-secretory epidermis was observed, as described in the following section.

Anatomical characterisation of the mature glands

Although the glandular epidermis of all species included in the sect. *Absus* subsect. *Baseophyllum* contained a non-secretory single-layered epidermis lacking stomata (Fig. 3*A*, *B*), two layers of cells were sometimes observed in the 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 forming at the margins of the glands (Fig. 3*A*, *B*). The epidermal cells at the margins were slightly columnar-shaped, showing thickened periclinal and anticlinal walls, with the outer periclinal

and anticlinal walls being much thicker than the inner periclinal walls (Fig. 3*C*). When a bi-layered epidermis was observed, the cells making up the second layer also contained thickened cell walls. Microchannels reaching the very top of the outer periclinal wall of the epidermal cells were observed through all the epidermal cells, being easily recognized at the margins of the glands (Fig. 3*C*), where the cells showed thicker walls.

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

Immediately below the secretory parenchyma tissue, there was a transition zone between the secretory parenchyma and the ground parenchyma cells (Fig. 3*E*, *F*). The transition zone was made up of 2–4 layers of polyhedral, lightly-stained cytoplasm, and highly vacuolated parenchyma cells which displayed larger volume than the parenchyma cells from the secretory tissue (Fig. 3*E*, *F*). Both idioblasts immersed in this zone and clusters of idioblasts below the transition zone were also observed.

Apart from *Chamaecrista cytisoides* var. *blanchetii*, which has sessile leaves (therefore providing the vascularisation by the pulvinus), the glands were vascularised by xylem and phloem cells (Fig. 3*F*, *G*, *H*) coming directly from the

vascular bundles of the petiole/rachis (Fig. 3*A*, *B*). However, the number of phloem cells outnumbered the amount of xylem cells (Fig. 3*F*). The accessory vascular bundles of some specimens of *C. cytisoides* var. *cytisoides*, *C. coriacea* and *C. cytisoides* var. *decora* also vascularised the glands (Fig. 3*I*). 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. *3E*, *F*). When going toward the glands, the cells surrounding the xylem and phloem differentiated into sclereids (Fig. 3*H*) instead of fibres. The sclereids extended to the parenchymatic zone right below the secretory tissue, forming a sheath of sclereids. Layers of fibres varied from 5–10 for the vascular bundles in the petiole/rachis, and 1–5 for the accessory vascular bundles (Fig. 3*A*, *I*). Numbers varied both among and within species.

In mature glands, a sheath of idioblast with prismatic crystals surrounding the layers of fibres around the accessory vascular bundles and the petiole/rachis vascular bundles was observed. Within the transition zone, idioblasts containing prismatic crystals forming a sheath immediately below the secretory tissue could also be found.

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.

A weak positive reaction with Sudan indicated lipids impregnating the walls of the secretory tissue cells and epidermal cells (Fig. 4*A*, *B*) in the glands of *C. cytisoides* var. *brachystachya*, *C. cytisoides* var. *cytisoides* and *C. cytisoides* var.

decora. Any distinction between the cuticle and other layers of the periclinal wall of the epidermal cells was not observed (Fig. 4*B*).

Total proteins grains were detected in the secretory tissue of *C. cytisoides* var. *blanchetii*, *C. cytisoides* var. *brachystachya*, *C. cytisoides* var. *decora* (Fig. 4*C*) and *C. cytisoides* var. *cytisoides* (Fig. 4*D*).

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

Lignins were detected in the walls of xylem cells, fibres and sclereids of *C. cytisoides* var. *brachystachya*, *C. cytisoides* var. *cytisoides*, *C. cytisoides* var. *decora* (Fig. 4*F*).

Neutral polysaccharides (Fig. 4*G*, *H*), acid muco-polysaccharides (Fig. 5*A*, *B*), pectins (Fig. 5*C*, *D*), and mucilages (Fig. 5*E*, *F*) 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. Neutral polysaccharides and pectins/mucilages (Fig. 5*D*) were clearly observed in the microchannels of the epidermal cells of *C. cytisoides* var. *blanchetii*, *C. cytisoides* var. *brachystachya*, *C. cytisoides* var. *cytisoides*, *C. coriacea* and *C. cytisoides* var. *decora*. Acid muco-polysaccharides and mucilages returned 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. cytisoides* var. *brachystachya*, *C. cytisoides* var. *cytisoides*, *C. coriacea* and *C. cytisoides* var. *decora*.

Strips for glucose identification did not react with the viscous exudates of *C. cytisoides* var. *blanchetii*, *C. cytisoides* var. *brachystachya* and *C. cytisoides* var. *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. cytisoides* var. *blanchetii*, *C. cytisoides* var. *brachystachya*, *C. cytisoides* var. *cytisoides* and *C. cytisoides* var. *decora* were exuded the most nectar under field conditions. Older leaves (i.e. seventh node on) were also observed to secrete, but did so rarely and less often than younger leaves. Ants visiting the secretion of the glands were also occasionally observed but not collected for identification.

Discussion

Based on the position, external morphology, anatomy, detection of glucose in the secretion and polysaccharides, these glands can be characterised as extrafloral nectaries (EFN). According to the classification proposed by Zimmermann (1932) for EFNs, the EFNs of *Chamaecrista* sect. *Absus* subsect. *Baseophyllum* may also be classified as 'Hochnektarien', which are elevated nectaries.

The EFNs present in *Chamaecrista* species of the subsect. *Baseophyllum* are structural nectaries according to Zimmermann's classification (1932). They are made up of an epidermis subtended by a specialised parenchymatous tissue, usually 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 were observed in the EFNs of the *Chamaecrista* species belonging to the subsect. *Baseophyllum*. Similar structures have been reported in literature, in Leguminosae genera as well as in other families (Durkee 1982; Pascal *et al.* 2000; Francino *et al.* 2006; Rocha *et al.* 2009).

The development pattern followed by the EFNs (i.e. from the protodermis and underlying layers) of the eight species of *Chamaecrista* studied in accordance with other reports for several plant families described in the literature (Elias and Gelband 1976; Durkee 1982; Paiva *et al*. 2007; Thadeo *et al.* 2008; Rocha *et al.* 2009; Coutinho *et al.* 2010). The asynchronous cellular division during the EFN ontogenesis has been reported by other authors (Dave and Patel 1975; Elias 1983; Thadeo *et al*. 2008; Rocha *et al*. 2009). At the second stage of development of some EFNs of *Chamaecrista cytisoides* var. *cytisoides*, the typical rounded area corresponding to the nectary primordium displayed a wrinkled, non-rounded surface. That may be the result of asynchronous divisions in the nectary primordium. The protodermal cells probably underwent more divisions than the ground meristem cells, which caused the wrinkling of the gland surface. In addition, some of the protodermal cells also divided periclinally, which in turn may have raised some areas in the protodermis and therefore resulted in its wrinkling.

In the *Chamaecrista* species studied, the presence of a single-layered epidermis made up of non-secretory cubical cells was found. This feature was observed in *Pithecellobium macradenium* (Leguminosae-Mimosoideae) by Elias (1972) and in *Chamaecrista trichopoda* by Francino *et al.* (2006), and this may be a

common taxonomic anatomical character for the family Leguminosae. However, little information on the role played by non-secretory epidermis is available. Conversely, the presence of a one or two-layered nectary epidermis made up of columnar-shaped cells is quite common among EFNs (1979a; Elias 1983; Nepi 2007; Paiva *et al.* 2007; Thadeo *et al.* 2008; Rocha *et al.* 2009; Coutinho *et al.* 2010).

As reported by several authors, the accumulation of secretion below a lifted cuticle (which later ruptures) is a general feature of EFNs and other glands (Elias *et al.* 1975; Fahn 1979a; McDade and Turner 1997; Nepi 2007; Thadeo *et al.* 2008; Rocha *et al.* 2009). 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 epidermises here were 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). A discontinuous cuticle has also been reported in septal nectaries (Nepi 2007). 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 *Chamaecrista 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 (Pacini *et al.* 2003; Nepi 2007). 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.

The presence of cell layers immediately below the secretory parenchyma tissue has been reported by other authors (Contreras and Lersten 1984; Francino *et al*. 2006; Nepi 2007). Contreras and Lersten (1984), Francino *et al*. (2006), and Paiva *et al.* (2007) reported the presence of thickened-wall cell layers subtending the secretory parenchyma. Nepi (2007) named this the parenchyma of the subnectariferous parenchyma. In *Cedrela fissilis* (Paiva *et al.* 2007), such thickenings are the result of the impregnation of the sheathing layer cell walls with lignin and suberin. According to these authors, this sheath acts as a barrier to apoplastic transport, preventing secretion from reflux to the inner tissues. 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 the 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 stored neutral polysaccharides, acid muco-polysaccharides, mucilage, and pectins. These substances might serve as an energy reservoir for nectar secretion as suggested by Fahn (1979b). In fact, the mucilage idioblast cells in the *Chamaecrista* species studied here were 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. Leitão *et al*. (2005)

hypothesised that the presence of mucilage cavities and ducts in contact with the vascular bundles close to floral and EFNs in *Triumfetta semitriloba* could be associated with a possible supply of water for secretion. Several authors have proposed that the mucilage is involved in water storage (Fahn 1979b; Sawidis 1991).

Vascularised nectaries comprising of both phloem and xylem ending below the nectariferous parenchyma have been reported in other works, as well as in Leguminosae (Pascal *et al.* 2000; Francino *et al.* 2006). Frey-Wyssling (Elias 1983) distinguished three types of vascularisation: exclusively by phloem, by phloem and xylem, and predominantly by xylem. As few tracheids were observed towards the EFNs, the vascularisation in the *Chamaecrista* species studied would fall into a forth group: predominantly by phloem, which was not reported by Frey-Wyssling. According to Carlquist (1969), the amount of vascular tissue in a structure is directly related to its size, instead of being related to the state of advancement, as proposed by Frey-Wyssling (1955). Our findings agree with Carlquist's proposition, as the EFNs in *Chamaecrista* sect. *Absus* subsect. *Baseophyllum* are conspicuous, large, and well structured, being frequently vascularised also by the accessory bundles.

The layers of fibres surrounding the vascular bundles were not present in the vascular tissue from the petiole/rachis or accessory bundles converged towards the EFNs. Instead, sclereids were observed to form a sheath right below the subnectariferous parenchyma. Such sclereids were not observed by Francino *et al.* (2006) in the EFNs of *Chamaecrista trichopoda*. 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. According to Evert (2006), sclereids are typically short

cells with thick secondary walls, strongly lignified, and provided with numerous simple pits, having as their principal function mechanical support. Therefore, sclereids may be acting in the mechanical support of the EFNs, as these EFNs are large and structured.

As observed in the *Chamaecrista* species studied here, prismatic crystals were also reported in *Chamaecrista trichopoda* (Francino *et al.* 2006). However, in *C. trichopoda* prismatic crystals were associated with only the vascular tissue in the petiole, not to the EFNs. Pascal *et al.* (2000) did not mention the presence of prismatic crystals in Caesalpinieae and Mimosoideae either. On the other hand, crystals are frequently mentioned as being present in different organs of Leguminosae plants (Watson 1981). Oxalate crystals have been observed by other authors studying nectaries in other species (Elias *et al.* 1975; Metcalfe and Chalk 1979; Elias 1983; Paiva and Machado 2006; Thadeo *et al.* 2008; Rocha *et al.* 2009; Coutinho *et al.* 2010). Ca precipitation as crystalliferous solid inclusions in the cells surrounding the veins would prevent Ca accumulation around chlorenchyma cells, which could affect cellular function (Franceschi and Nakata 2005). Because sucrose transport in plants involves ATPase activity (Giaquinta 1979) and Ca^{2+} inhibits plasma membrane ATPase (Leonard and Hodges 1980), crystals may immobilize calcium in the nectary region where active sugar transport is at play (Vezza *et al.* 2006), guaranteeing the continuous transport of sucrose to the nectary tissues.

The presence of mucilage, pectins, proteins, and polysaccharides in the nectariferous parenchyma of EFNs may be an indication that they might be found in the exudates, which is evidence the nectar's complexity. The presence of such substances in EFNs has also been described by other authors (O'Dowd 1979;

Caldwell and Gerhardt 1986; Koptur 1994; Leitão *et al.* 2005; Francino *et al.* 2006; 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 (Roshchina and Roshchina 1993; Nicolson and Thornburg 2007). In a similar way, we believe that the presence of phenolic compounds in the EFNs 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).

It has long been shown that EFNs may be engaged in defensive strategies based on ant aggressiveness against herbivores (Bentley 1977; O'Dowd 1979; Elias 1980; McKey 1989; Oliveira 1997; Heil *et al.* 2000; Kost and Heil, 2005), which may help to increase the amount of fruit set (Oliveira *et al.* 1999) and may even adapt nectar flow to nectar-feeding ant consumption (Heil *et al.* 2000). In our study, the presence of total protein grains was found by using the dye xylidine Pounceau. We believe that proteins may be hydrolysed into amino acids which then became 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) have shown that amino acid supplementation in extrafloral nectars resulted in a significant decrease in the mass of male crickets consumed (i.e. crickets act as a natural source of proteins to ants); that is, ants preferred extrafloral nectars with amino acids to the crickets offered. 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. If so, over

evolutionary time ants may favour plants that produce nectars with high levels of amino acids (Lanza 1991). On the other hand, the presence of many small nectaries at a particular site has been considered a form of specialisation by Elias and Gelband (1976), as malfunction or damage in one or two of these small nectaries will not eliminate the attraction of ants to that particular site for other small nectaries that are present. Conversely, the EFNs in *Chamaecrista* subsect. *Baseophyllum* are vascularised, large, and may secrete nectar amounts that compensate for their limited abundance per leaf.

Secreting EFNs of young developing leaves and newly expanded leaves of *C. cytisoides* var. *blanchetii*, *C. cytisoides* var. *brachystachya*, *C. cytisoides* var. *cytisoides* and *C. cytisoides* var. *decora* under field conditions were observed to exude more nectar than older leaves. We believe that the role of the nectaries is related to ant/plant associations, but because ants might be involved in the protection of fragile young unfolding leaves that are more susceptible to herbivores, as proposed by several works (Heil *et al.* 2000; Leitão *et al.* 2005; Paiva *et al.* 2007; Coutinho *et al.* 2010), which concur 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). Also, young leaves may lack other types of defences against herbivores, such as secondary metabolites, and effective mechanical tissues such as fibres (Harper 1989). Young leaves are generally important for future plant fitness since they already have caused high construction costs, acting as sink organs, without having contributed much yet to the plant's pool of photoassimilates (Radhika *et al.* 2008). Hence, they still have the

highest future life expectancy and can therefore contribute plenty to the prospective photosynthetic assimilation (Radhika *et al.* 2008).

EFNs found on older leaves were generally not secreting, and on the rare occasions when secretions were found, only small amounts were observed in comparison to the EFNs on young secreting leaves. As ants have to climb up the plants in order to find the secreting EFNs on the young leaves at the top of the crown, they may also patrol the older leaves which do not display secreting EFNs. Obviously, ants may patrol much less on older leaves, but these leaves would still be patrolled. In fact, during field observations we observed older leaves being patrolled by ants.

The EFNs are on the adaxial side of the leaf are completely exposed to environmental conditions such as sunlight and wind, which may increase nectar viscosity. As such, viscous nectar was observed in the field. It is certain that the exudates did not readily react with the glucose identification strips, as they had to be diluted first. Increasing nectar viscosity may decrease nectar evaporative loss. However, it may also affect ant/plant interactions. According to Josens *et al*. (1998), crop load increases with increasing sucrose concentration until a maximum of about 43%, when it diminishes because of nectar viscosity, suggesting that the physical properties of the solution determines the rate of fluid ingestion. This may alter both the interest of ants in the extrafloral nectaries and the time spent on the collections, which could affect ant patrolling and therefore the plant protection.

In addition to attracting ants, other possible physiological and ecological functions have been suggested, such as the release of excess sugars (Frey-Wyssling 1955; Mound 1962). Indeed, *Chamaecrista* species are, as a whole, found in sunny

areas and a photosynthetic surplus of sugars may be expected. Then, we could anticipate that older leaves would keep secreting nectar throughout their life time or at least secrete more nectar than younger leaves, as a way to get rid of the surplus sugars produced. However, this is not what we observed. If EFNs were in fact involved in the release of excess sugars, we would expect the EFNs on younger leaves to secrete less nectar than older leaves, not the opposite as observed in the field. As shown by Radhika *et al.* (2008), younger leaves exuding nectar act as sink organs.

Although the position of the EFNs on the petiole/rachis may aid the identification of some of the species included in the subsect. *Baseophyllum*, the EFNs are anatomically and morphologically similar. As a consequence, these glands could not be used in the clear delimitation of species. However, they can still have promising taxonomic value as they are considered a conservative character, unifying the subsect. *Baseophyllum* as a monophyletic group. The importance of EFNs in the subgeneric delimitations has been recorded for the genus *Cassia* Linn. (Bhattacharyya and Maheshwari 1971), which is close to *Chamaecrista* (Laxmikanta *et al.* 2010).

The EFNs found in *Chamaecrista* sect. *Absus* subsect. *Baseophyllum*, constitute a synapomorphy for this subsection, as they are absent in the other species belong to *Chamaecrista* sect. *Absus*. It is also relevant to highlight the correlation between the presence of the EFN in *Chamaecrista* sect. *Absus* subsect. *Baseophyllum* with the absence of the sticky glandular trichomes, because if the secretion produced by the trichomes were present it would catch the EFN visiting ants.

We believe that EFNs in genus *Chamaecrista* have a single origin, as similarities morphological among the EFNs of *Chamaecrista* species bearing EFNs may be observed (Francino 2006; Francino *et al.* 2006; Conceição *et al.* 2009). In additional, although the EFNs studied by Francino (2006) and Francino *et al.* (2006) present a stalk, these glands show anatomical similarities with the EFNs of *Chamaecrista* sect. *Absus* subsect. *Baseophyllum*.

Conceição *et al.* (2009) has shown that EFNs were already present in species of *Chamaecrista* sect. *Apoucouita*, which is at the base of the author's phylogenetic tree. This suggests that EFNs were present in the ancestor of the subtribe Cassiinae and were transferred to the sections *Apoucouita*, *Caliciopsis*, *Chamaecrista* and *Xerocalyx*, but were lost in the lineage which gave rise to the sections *Grimaldia* and *Absus*, later re-appearing in the subsect. *Baseophyllum* (which belongs to sect. *Absus*).

The molecular study of the subtribe Cassiinae performed by Laxmikanta *et al.* (2010) placed *Chamaecrista* at the base of the dendrogram, with *Cassia* and *Senna* deriving from *Chamaecrista*. This suggests that the EFNs were lost by *Cassia* and the first lineages of *Senna*, and re-appeared as a synapomorphy for the advanced lineages of *Senna*, as suggested by Marazzi *et al*. (2006). In fact, Marazzi *et al.* (2006) has rejected the hypothesis that EFNs are an "archaic feature" for *Senna*. Moreover, their phylogenetic results have clearly shown that all sampled species with EFNs formed a strongly supported clade, indicating that the EFNs in *Senna* appear to have evolved only once, constituting a clear synapomorphy for the clades bearing EFNs.

The hypothesis on the gain and loss of EFNs is supported by Bowman and Smyth's (1999) work, which proved that there is a gene which regulates nectary development in flowers. It is possible that a similar gene may be responsible for the development of the EFNs. This gene could also work in conjunction with other genes which activate or deactivate the expression of the gene responsible for the EFN development. That may be the reason why EFNs are gained and lost in species belonging to the subtribe Cassiinae, representing an interesting model for studies on the gene expression of EFNs.

Conclusion

The position, external morphology, anatomy, detection of glucose in the secretion, polysaccharides and other substances allow us to characterise these glands as extrafloral nectaries. Although the EFNs may differ regarding their size and position, they are anatomically and morphologically similar, acting as a conservative unifying character for sect. *Absus* subsect. *Baseophyllum*, revealing promising taxonomic value for this subsection. Further studies comparing the EFNs present in species studied here with other *Chamaecrista* will lead to interesting and more conclusive approaches. Further specific ecological studies on the role of the EFNs in *Chamaecrista* sect. *Absus* subsect. *Baseophyllum* are required for clearer ecological conclusions.

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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 cytisoides* var. *blanchetii*, *C. cytisoides* var. *brachystachya*, *C. coriacea*, *C. cytisoides* var. *cytisoides*, and *C. cytisoides* var. *decora* with their respective location used in this study. Acronyms for Brazilian States: ES - Espírito Santo; MG - Minas Gerais.

Irwin and Barney (1982)	**Herbaria**	**Fresh Material**		**Vegetation type where the species are found**
		Municipality	**Location**	
C. coriacea	HUEFS 112.739 SPF 79.573	Conceição do Mato Dentro - MG (Gurutubas-Costa Sena)	18º43'31"S, 43º37'24"W	campo rupestre
C. depauperata Conceição *et al.* (2001)	HUEFS 94.669 HUEFS 112.632 SPF 79.580	-----	-----	campo rupestre
C. cytisoides var. *blanchetii*	HUEFS 66.389 HUEFS 131.380 SPF 90.113	Guarapari – ES (Parque Estadual Paulo César Vinha)	20º 35'7.8"S, 40º25'17.3"W	campo rupestre, cerrado, restinga
C. cytisoides var. *brachystachya*	HUEFS 12.469 HUEFS 17.060 SPF 179.567	Diamantina – MG (7 km East from Diamantina)	18º10'54"S, 43º33'45.5"W	caatinga,campo rupestre, cerrado, restinga
C. cytisoides var. *confertiformis*	HUEFS 65.042 HUEFS 65.038 SPF 179.572	-----	-----	campo rupestre
C. cytisoides var. *cytisoides*	HUEFS 105.351 HUEFS 71.567 SPF 160.397	Santa Bárbara do Monte Verde - MG (Três Cruzes-Serra Negra)	21º58'0.3"S, 43º49'12.1"W	transition between campo rupestre and fog forest (cloud forest)
C. cytisoides var. *decora*	HUEFS 75.078 SPF 42.876	Diamantina –MG (Biribiri)	18º08'53.2"S, 43º36'53.1"W	campo rupestre
C. cytisoides var. *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. cytisoides* var. *blanchetii*; 2 = *C. cytisoides* var. *brachystachya*; 3 = *C. cytisoides* var. *coriacea*; 4 = *C. cytisoides* var. *cytisoides*; 5 = *C. cytisoides* var. *decora*

Metabolic Group		Histochemical Test	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

Table 3. *Chamaecrista s*pecies and respective gland position, number, and shape.

Species	Position	Number	Shape
C. cytisoides var. *blanchetii* (Fig. 1*A*)	Between the single pair of leaflets	1	Rounded/elliptical concave or flat triangular shaped
C. cytisoides var. *cytisoides*	On the interfoliolar segments of the rachis	1–2	Rounded/elliptical concave
C. coriacea	Near the middle of the petiole	1	Rounded/elliptical concave
C. cytisoides var. *decora* (Fig. 1*B*)	Mostly near the middle of the petiole. Few glands are place below the upper pair of leaflets	1	Rounded/elliptical concave
C. cytisoides var. *brachystachya*	On the interfoliolar segments of the rachis or between the lower pair of leaflets	1–2	Rounded/elliptical concave
C. cytisoides var. *confertiformis*	Below the upper pair of leaflets	1	Rounded/elliptical concave
C. depauperata	Middle of the petiole	1	Rounded/elliptical concave
C. cytisoides var. *unijuga*	Middle of the petiole	1	Rounded/elliptical concave

Fig. 1. (*A*) *Chamaecrista cytisoides* var. *blanchetii*. In detail, the gland found between the leaflets covered with secretion. (*B*) *C. cytisoides* var. *decora*. In detail, the petiolar gland also covered with secretion.

Fig. 2. Cross sections of the rachis showing the ontogenetic process of the gland. (*A-D*) *Chamaecrista cytisoides* var. *brachystachya*. (*E–J*) *C. cytisoides* var. *cytisoides*. Initials: Ab, accessory bundle; Cl, layers of cells surrounding the accessory bundle; Ep, epidermis; Gm, ground meristem; Gp, gland primordium; Pc, procambium; Ph, phloem; Pr, protodermis; Sp, secretory parenchyma; Xy, xylem; Va, vascularisation; asterisk, sclereid; arrow, meristematic cell.

Fig. 3. Glands found on the petiole/rachis of *Chamaecrista* species. Cross (*A, C–I*) and longitudinal (*B*) sections of the petiole/rachis. (*A, B, H, I*) Field collected material embedded in histological paraffin. (*C, E, F, G*) Herborized material embedded in historesin. (*D*) Sections of fresh non-treated samples. (*A, H*) *C. cytisoides* var. *brachystachya.* (*B, D*) *C. cytisoides* var. *decora*. (*C*) *C. cytisoides* var. *blanchetii*. (*E, G*) *C. cytisoides* var. *confertiformis*. (*F*) *C. depauperata*. (*H*) *C. coriacea*. Initials: Ab, accessory bundle; Ep, epidermis; Fi, fibres; Id, idioblast; Ph, phloem; Sp, secretory parenchyma; Tz, transition zone; Va, vascularisation; Xy, xylem; asterisk, spaces among the cells, black arrow, transition zone; white arrow, sclereid.

Fig. 4. Histochemical tests of glands found on the petiole/rachis of *Chamaecrista* species. Cross (*A–D, F–I*) and longitudinal (*E*) sections of the petiole/rachis. Field

collected material embedded in histological paraffin. (*C, F*) Sections of fresh samples. (*A–C, F–I*) *C. cytisoides* var. *decora.* (*D*) *C. cytisoides* var. *cytisoides*. (*D*) *C. cytisoides* var. *brachystachya*. (*E*) *C. cytisoides* var. *brachystachya*. (*A, B*) Sudan IV. (*C, D*) Xylidine Pounceau. (*E*) Fixation with ferrous sulphate in formalin. (*F*) Phloroglucinol. (*G, H*) Periodic Acid Schiff. (*H*) Note the cluster of idioblasts found at the phloem endings. Initials: Ab, accessory bundle; Ep, epidermis; Id, idioblasts; Sc, sclereids; Sp, secretory parenchyma; Tz, transition zone; Va, vascularisation; Xy, xylem; asterisk, secretion among cells; black arrow, transition zone.

Fig. 5. Histochemical tests of glands found on the petiole/rachis of *Chamaecrista* species. (*A–F*) Cross sections of the petiole/rachis. (*A–D, F*) Field collected material embedded in histological paraffin. (*E*) Sections of fresh samples. (*A*) *C. cytisoides* var. *brachystachya.* (*B*) *C. cytisoides* var. *blanchetii.* (*C–F*) *C. cytisoides* var. *decora*. (*A, B*) Alcian blue. (*C, D*) Ruthenium red. (*E, F*) Tannic acid/ferric chloride. Initials: Ep, epidermis; Id, idioblasts; Ph, phloem; Sp, secretory parenchyma; Tz, transition zone; Xy, xylem.

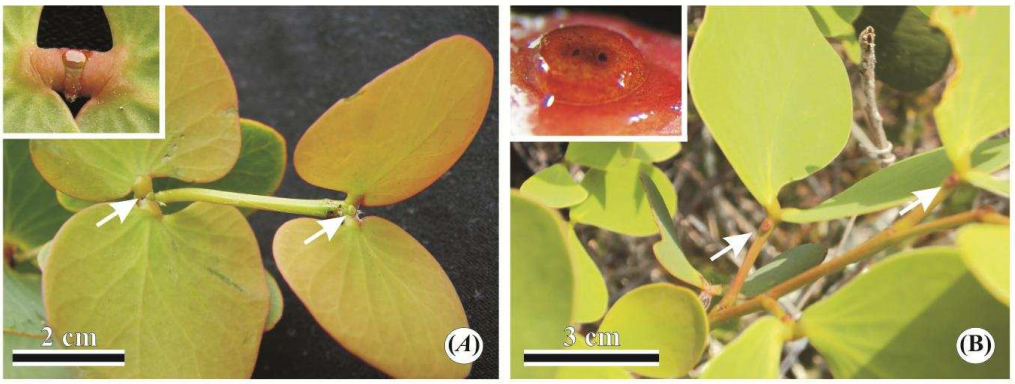
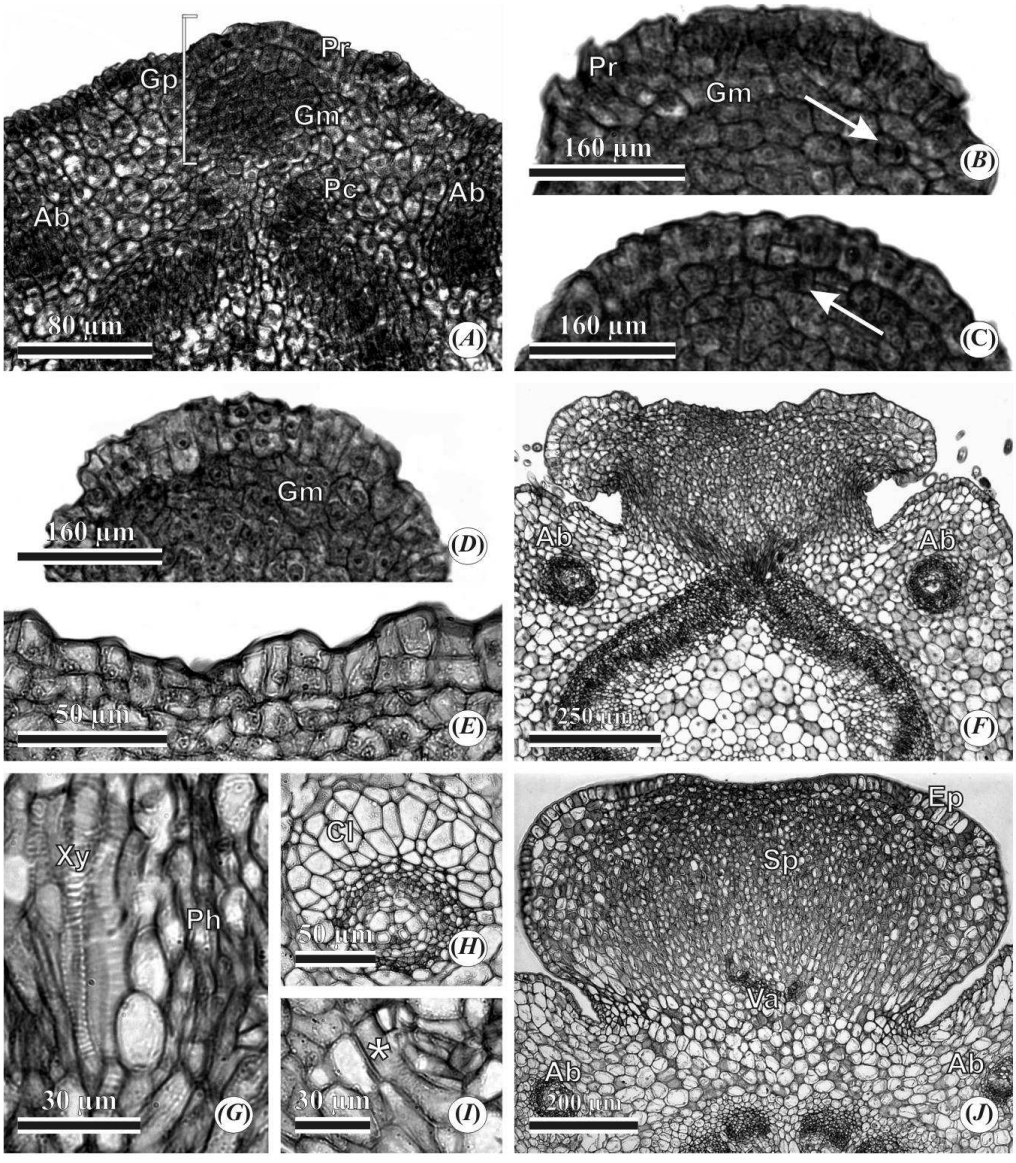


Figure 1


Pr
Gp
Gm
Pc
Ab
Ab
80 µm
(A)
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(D)
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Figure 2

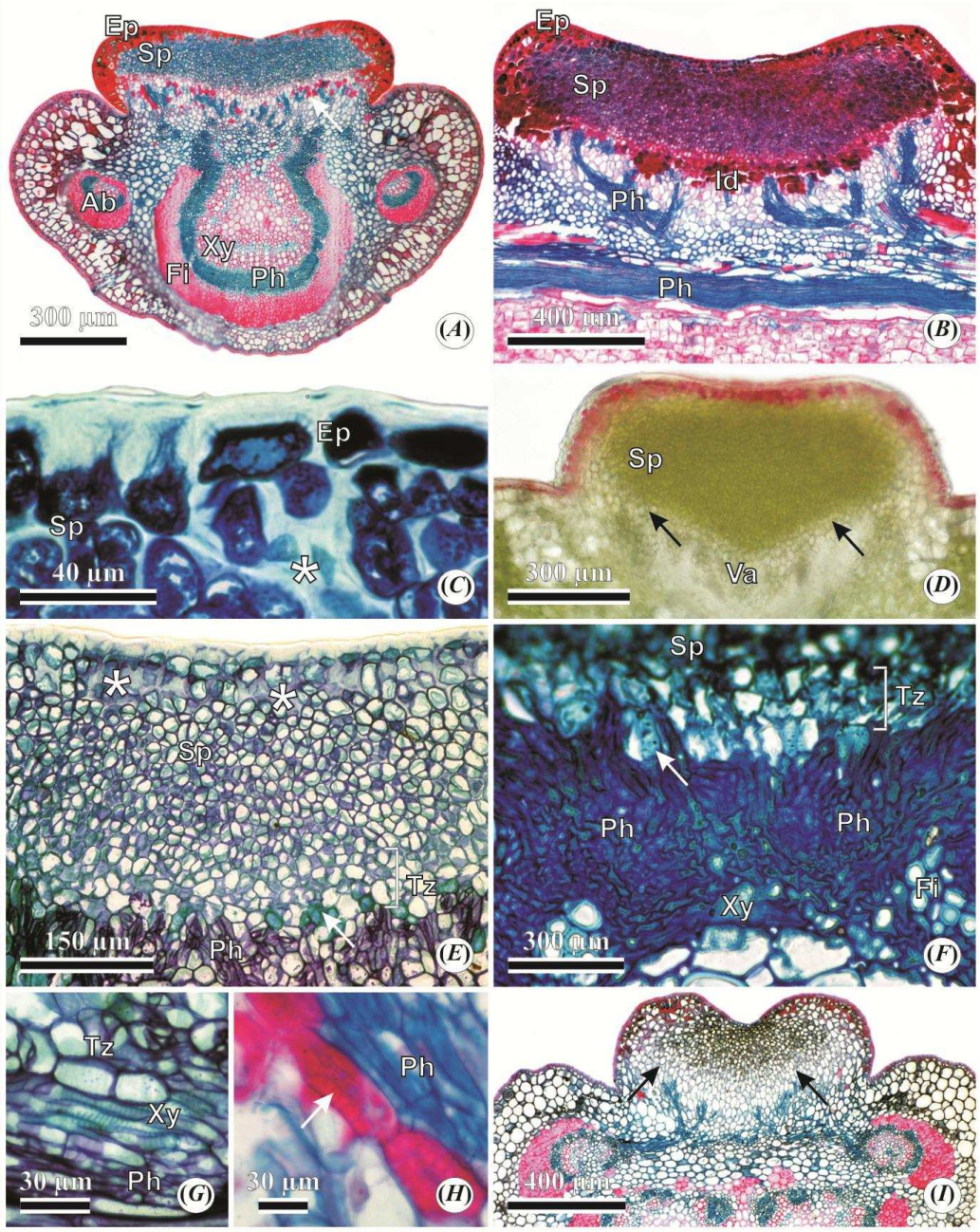
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Figure 3

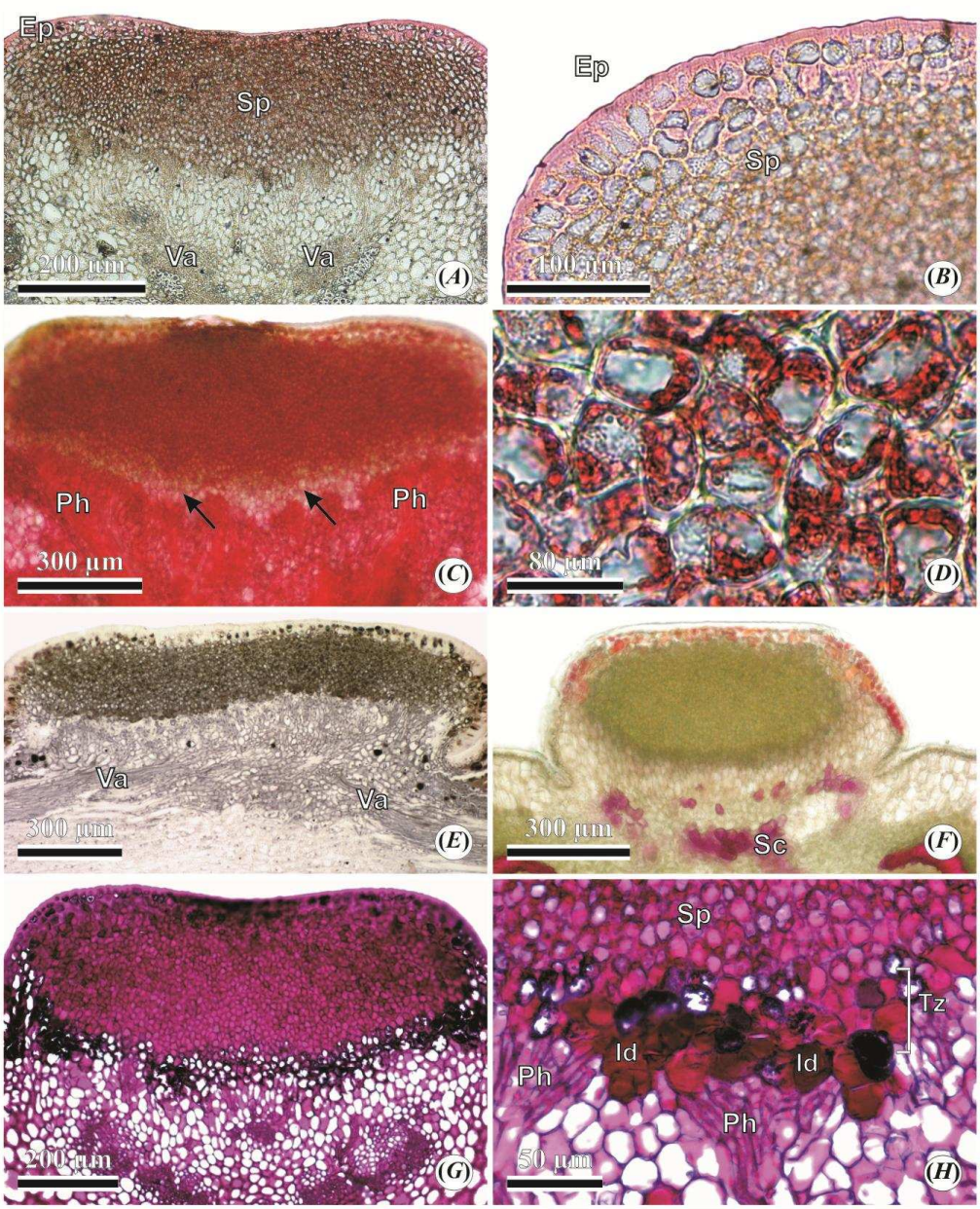
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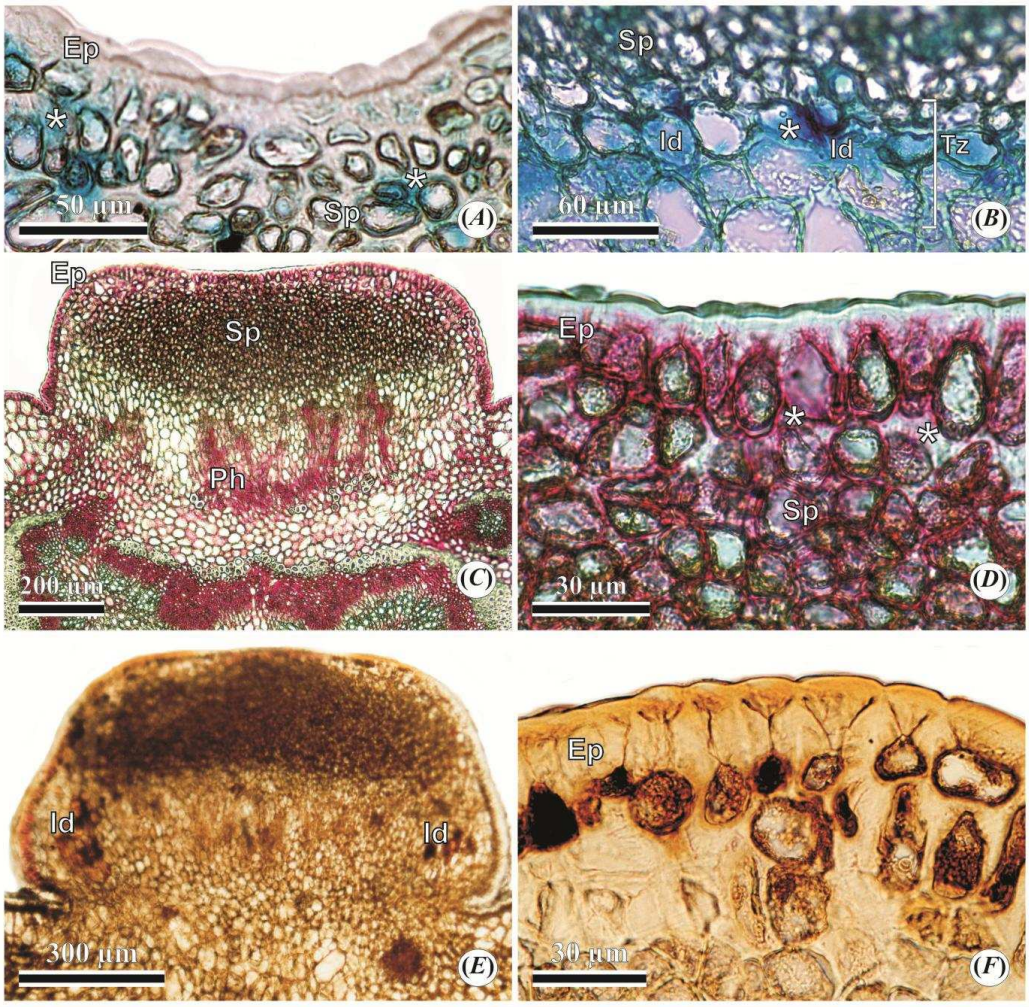
Figure 4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(C)
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(D)
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Id
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(E)
Ep
30 µm
(F)

Figure 5

Artigo 2

Leaf anatomical studies as an additional taxonomy tool for *Chamaecrista* section *Absus* subsection *Baseophyllum* (Leguminosae, Caesalpinioideae)

Leaf anatomical studies as an additional taxonomy tool for *Chamaecrista* section *Absus* subsection *Baseophyllum* (Leguminosae, Caesalpinioideae)

Ítalo Antônio Cotta Coutinho [1], Dayana Maria Teodoro Francino [1], Aristéa Alves Azevedo [1], Renata Maria Strozi Alves Meira[1]

[1]Universidade Federal de Viçosa (UFV), Departamento de Biologia Vegetal, Viçosa 36570-000, Minas Gerais, Brazil.

Corresponding author: Renata Maria Strozi Alves Meira (rmeira@ufv.br)

Abstract

This study aims at answering which anatomical and morphological characters of the leaflets and petiole/rachis may be used on the separation of the varieties of *Chamaecrista cytisoides* (section *Absus* subsection *Baseophyllum*) into species, as defined by recent molecular studies. Anatomical and morphological characters which are useful to distinguish these varieties into species and also to unite them as a monophyletic group are given. All eight species of *Chamaecrista* sect. *Absus* subsect. *Baseophyllum* displayed a single-layered epidermis. Actinodromous-camptodromous-brochidodromous venation type, collateral vascular bundles arranged in open arc, layers of fibres and a sheath of cells containing prismatic crystals surrounding the vascular bundles, enlarged tracheids at the vein endings, vascular tissue of petiole/rachis with a pith parenchyma, accessory bundles, extrafloral nectaries, colleters, and non-secretory trichomes were observed in all the species studied and considered as unifying characters for this subsection. Position and type of stomata on leaves, type of mesophyll, number of both palisade and spongy parenchyma layers, presence of hypodermis, position of mucilage idioblasts in the leaflets and petiole/rachis, and number of accessory bundles were used to make distinction among the species. Principal component analysis (PCA) based on morphological and anatomical characters showed the clear separation of the eight species included within sect. *Absus* subsect. *Baseophyllum*, corroborating the results achieve by molecular analyses.

Keywords: "campo rupestre", colleter, extrafloral nectaries, mucilage idioblasts, secretory structures, hypodermis.

Introduction

Leguminosae is a large family with more than 19,300 species distributed mainly in the tropical areas of the Americas, Africa and Asia (Lewis *et al*. 2005). Several Leguminosae species have long been used in the agriculture for the production of fodder and human food, in the wood industry, pharmacology, soil fertilization, landscaping, and so on (Graham and Vance 2003; Lewis *et al*. 2005).

The genus *Chamaecrista* in Brazil, is found in the ‘‘campos rupestres’’ vegetation (‘rocky fields’), in the ‘‘cerrados’’ (savannah-like vegetation), in the coastal ‘‘restingas’’ vegetation (sandy coastal plain), and in the ‘‘caatingas’’ vegetation (seasonally dry thorny forest) (Irwin and Barneby 1978; Conceição 2006; Conceição *et al*. 2008).

Chamaecrista Moench is one of the largest genera of this family. The genus *Chamaecrista* was named after the Greek word *chamae* (dwarf) and the latin word *crista* (a crest), the latter in allusion to *Caesalpinia crista* L., most probably because of the similarity of its group of stamens to a crest (Pedley 1998). *Chamaecrista* species are herbs or shrubs, with leaves paripinnate; opposite leaflets; strongly nerved; persistent stipules; petiole usually bearing a concave extrafloral nectaries (EFN); additional EFNs are sometimes present on petiole or leaf rachis. As the other species included in the subtribe Cassiinae, they bear floral characters interpreted as specializations to buzz pollination (absence of floral nectaries, and poricidal anthers) (Irwin and Barneby 1982; Marazzi *et al*. 2006).

Although created by Moench in 1794 (Pedley 1998), *Chamaecrista* has a complex taxonomic history. It was formerly included in the genus *Cassia* (Bentham

1870; Bentham 1871; Irwin and Barneby 1978). The segregation of *Chamaecrista* from *Cassia* was consolidated by Irwin and Barneby's work in 1982.

According to Irwin and Barneby (1982), the genus is sorted out into six sections: *Chamaecrista* section *Apoucouita*, *Absus*, *Caliciopsis*, *Chamaecrista*, *Grimaldia*, and *Xerocalyx*. One of the most distinct characters of these sections is the presence of sticky glandular hairs (SGH) and absence of extrafloral nectaries (EFN) on the petiole and/or rachis of species belonging to the sections *Absus* and *Grimaldia*, and the absence of SGHs and presence of EFNs in extrafloral nectaries in the species of the sections *Apoucouita*, *Caliciopsis*, *Chamaecrista*, and *Xerocalyx* (Irwin and Barneby 1982). *Chamaecrista* sect. *Absus* is further divided into four subsections (Irwin and Barneby 1982): *Chamaecrista* sect. *Absus* subsection *Absus*, *Adenophyllum*, *Baseophyllum*, and *Otophyllum*.

The following characters lead Irwin and Barneby (1978) to include subsection *Baseophyllum* within *Chamaecrista* sect. *Absus*: inflorescence simple terminal racemose, or racemose-paniculate or axillary racemose; perennial; commonly woody or functionally herbaceous from a xylopodium; phyllotaxy of mature stems spiral; androecium 10-merous; style cylindrical; and minute stigma symmetrically terminal, poriform. Although included in the sect. *Absus*, the species of the subsect. *Baseophyllum* do not bear the typical sticky glandular hairs of this section, but display extrafloral nectaries on the petiole/rachis, which are supposed to be lacking (Irwin and Barneby 1982; Conceição *et al*. 2008; Conceição *et al*. 2009).

The most used taxonomic classification for the subsect. *Baseophyllum* includes only two species: *Chamaecrista coriacea* (Benth.) H.S.Irwin & Barneby and *C. cytisoides* (Collad.) H.S.Irwin & Barneby, with *C. cytisoides* including seven

varieties. The floral characteristics of the subsect. *Baseophyllum* are not strong enough to make distinction among these species and varieties (Irwin and Barneby 1978). As a result, they had to use weak morphological features to differentiate these species and varieties. Similar problems were faced by Conceição *et al*. (2008) when using morphological and floral characters on canonical variate analysis.

Much effort involving molecular studies contributing to a comprehensive phylogeny of legumes has been devoted to the taxonomic problems regarding Leguminosae in the past 10 years (Doyle et al. 2000; Bruneau et al. 2001; Wojciechowski et al. 2004; Bruneau et al. 2008; Conceição et al. 2008; Conceição et al. 2009; Hamza et al. 2009; Laxmikanta et al. 2010). The phylogeny of *Chamaecrista* has been performed by Conceição et al. (2009). Besides supporting the up-ranking of the subsect. *Baseophyllum* to the sectional status (Conceição et al. 2009), data on the molecular phylogeny of this subsection (Conceição et al. 2008) support the recognition of eight species: *Chamaecrista blanchetii* (Benth.) Conc., L.P.Queiroz & G.P.Lewis, *C. brachystachya* (Benth.) Conc., L.P.Queiroz & G.P.Lewis, *C. coriacea* (Benth.) H.S.Irwin & Barneby, *C. confertiformis* (H.S.Irwin & Barneby) Conc., L.P.Queiroz & G.P.Lewis, *C. cytisoides* (Collad.) H.S.Irwin & Barneby, *C. decora* (H.S.Irwin & Barneby) Conc., L.P.Queiroz & G.P.Lewis, *C. depauperata* Conc., L.P.Queiroz & G.P.Lewis, and *C. unijuga* (Benth.) Conc., L.P.Queiroz & G.P.Lewis.

Due to difficulties on distinguishing genera and species based only on morphological and floral characters, especially when varieties of a species are involved, taxonomists have also looked for other taxonomy tools, such as anatomical

characters, which may give support to species identification (Solereder 1908; Metcalfe and Chalk 1950, 1979; Araújo et al. 2010; Saheed and Illoh 2010).

This study aims at answering which anatomical and morphological characters of the leaflets and petiole/rachis may be used on the separation of the *Baseophyllum* species defined by the molecular studies, corroborating the taxonomic re-arrangement proposed by Conceição et al. (2008), that is, the distinction of eight species within the subsect. *Baseophyllum* instead of being treated as varieties of *C. cytisoides*. A dichotomous anatomical key will be generated for separation of the species. We also intend to confirm the monophyly of the subsect. *Baseophyllum* by providing anatomical and morphological characters which may group this species together. We will also discuss on the anatomical characters which may be enabling the survival of these eight species to survive at harsh environments such as the "campos rupestres", "caatingas" and "restingas".

Material and Methods

Plant material

Samples of petioles, rachises and leaflet blades of fully expanded leaves of all eight species of *Chamaecrista* belonging to the sect. *Absus* subsect. *Baseophyllum* as proposed by Conceição *et al.* (2008). Samples of both voucher and fresh materials of *C. blanchetii*, *C. brachystachya*, *C. coriacea*, *C. confertiformis*, *C. cytisoides*, *C. decora*, *C. depauperata*, and *C. unijuga* were used in the study carried out (Table 1). Field expeditions were based on consultation of voucher material cards and on coordinates provided by Conceição *et al*. (2008).

Slides preparation

Samples from the middle area of the leaflet (middle vein, margin, and area between the margin and the middle vein) were used for the structural characterisation.

The vascular arrangement of the petiole was studied right after the insertion of the first pair of leaflets in an attempt to standardise our data as not all leaves of the eight species are charged with a petiole. On the other hand, leaves of *C. decora*, *C. depauperata* and *C. unijuga* rarely have rachises so samples from the petiole right before the insertion of the single pair of leaflet. As *C. blanchetii* has sessile leaves samples of the petiole/rachis could not be taken.

For the anatomical characterisation, samples of voucher materials were rehydrated by boiling in distilled water (Smith and Smith 1942). After hydration, samples were dehydrated in ethylic series, stored in 70% ethanol, and embedded in methacrylate (Historesina Leica, Leica Microsystems Nussloch Gmbh, Heidelberg) according to Meira and Martins (2003). Blocks were sectioned with an automatic rotary microtome (Leica RM2155, Deerfield, IL, USA) at 4 µm thick using a glass knife. Sections were stained with toluidine blue at pH 4.0 (O'Brien and McCully 1981), dried at room temperature for 24h, and mounted in resin (Permount, Fisher Scientific, NJ, USA).

Samples from five species (*C. blanchetii*, *C. brachystachya*, *C. coriacea*, *C. cytisoides*, and *C. decora*) recollected in the field in April, October and November 2010 were also subjected to histochemical tests. When fertile, voucher materials of recollections were deposited at the herbarium of the Universidade Federal de Viçosa (VIC). Fresh material was fixed FAA (formaldehyde, acetic acid and 50% ethyl alcohol; 1:1:18, v/v) for 48 h, stored in 70% ethanol, dehydrated through a tert-butyl

alcohol series, and embedded in histological paraffin (Histosec®, Merck, Germany) (Johansen 1940). Cross, longitudinal and paradermal serial sections at 7 µm thick were obtained from blocks sectioned using a rotary microtome (Spencer 820 American Optical Corporation, Buffalo, NY, USA) and stainless steel blade. Sections were deparaffinised in xylene, brought to water through a ethyl alcohol series decreasing in concentration stained with safranin and astra blue, dehydrated through an ethyl alcohol/xylene series (Roeser 1972), and mounted in resin (Permount).

Whole mature leaflets of fresh and voucher material were diaphanised using 10% sodium hydroxide solution and 20% hypochlorite (Johansen 1940), stained with very diluted fuchsin (a drop of 1% fuchsin in 50% ethanol for each 10mL of distilled water) (Johansen 1940, modified), and mounted in glycerinated gelatin. The classification of the venation types, stomata types, and vascular arrangement of the petiole/rachis were performed according to Hickey (1979), Wilkinson (1979), Howard (1979), respectively. First, second and third node leaflets of fresh material of *C. cytisoides* were also diaphanised for elucidating the developmental pattern of the stomata. Diaphanisations were also used to investigate the presence of colleters which were classified according to Lersten (1974).

For the description of the epidermal surface and also stomata types, samples from leaflets were dissociated with 10% nitrous acid and 10% chromic acid (v/v) (Jensen 1962). Fragments produced by this procedure were stained with diluted astra blue (1 drop of 2% aqueous astra blue for each 10mL of distilled water) and mounted in glycerinated gelatin.

Histochemical tests

After deparaffinisation and hydration, as described in the slides preparation section, the following histochemical tests were employed in cross sections of leaflets: for total proteins, xylidine pounceau (O'Brien and McCully 1981); for total polysaccharides, periodic acid Schiff (PAS) (Maia 1979); for acid muco-polysaccharides, alcian blue (Pearse 1980); for pectins, ruthenium red (Johansen 1940); for mucilage, tannic acid/ferric chloride (Pizzolato and Lillie 1973). For detection of general phenolic compounds, fresh samples were fixed in a solution of ferrous sulphate in formalin (Johansen 1940). Sections of fresh samples made on a LPC table microtome (Rolemberg and Bhering Comércio e Importação LTDA, Belo Horizonte, Brazil) were used for detection of lignins with phloroglucinol (Johansen 1940) and lipid compounds with sudan IV in 70% ethanol (Pearse 1980).

Image and photographic documentation

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).

Phenetic analysis

Morphological data of species not observed in the field (*C. unijuga, C. depauperata* and *C. confertiformis*) were taken from Conceição *et al.* (2006).

A total of 54 anatomical and morphological characters (Appendix 1) were selected for the phenetic study. The matrix with species *vs.* anatomical and

morphological characters (Appendix 2) was first created in the Microsoft Excel program Version 11.0 and then exported to the PC-ORD program Version 5.10. The multivariate method with principal component analysis (PCA) was carried out.

Results

Venation Pattern

A typical camptodromous or actinodromous venation type could not be found in none of the eight *Chamaecrista* species studied. At the base leaves displayed the actinodromous venation type (Fig. 1*A*) and towards the margins leaves were camptodromous-brochidodromous (Fig. 1*B*, *C*), being therefore classified as actinodromous-camptodromous-brochidodromous.

The number of vascular bundles arising from the leaflet base varied among samples of the same species, and more samples are necessary to confirm this data. As a consequence, we did not include this information in our study.

Typical looped marginal ultimate venation was found in *C. coriacea* (Fig. 1*B*), while typical incomplete marginal ultimate venation in *C. depauperata* (Fig. 1*C*). Both looped and incomplete marginal ultimate venations were observed in *C. blanchetii*, *C. brachystachya*, *C. confertiformis*, *C. cytisoides*, *C. decora* and *C. unijuga*.

Leaflet Epidermis

Whole leaflets clearings revealed wave-shaped epidermal cells with slightly sinuous anticlinal walls on both leaflet blade sides of *C. confertiformis* only (Fig. 1*D*). Straight anticlinal walls were observed on the adaxial and abaxial epidermis of the other species (Fig. 1*E–L*).

Apart from *C. confertiformis*, which had amphistomatic (Fig. 2*A*) leaves and anisocytic stomata (Fig. 1*D*), all species had epistomatic leaves (Fig. 2*B–D, F, G*) and paracytic laterocyclic stomata (Fig. 1*E–I*).

Typical paracytic laterocyclic stomata with two cells with one cell bigger than the other surrounding the stomata in a circle-like pattern was observed in *C. brachystachya* (Fig. 1*F*), *C. confertiformis*, *C. coriacea* (Fig. 1*E*), *C. depauperata* and *C. unijuga*. Modifications of typical paractytic laterocyclic types were observed in *C. blanchetii*, *C. brachystachya*, *C. cytisoides*, *C. decora* and *C. depauperata*. As observed for *C. cytisoides,* the subsidiary cells of the paracytic laterocyclic stomata divided in a variety of planes, originating several types of modified paracytic laterocyclic stomata (Fig. 1*J–L*). Paracytic laterocyclic stomata with one the two cells dividing anticlinally in relation to the guard cell originating anisocytic-like stomata was observed in *C. blanchetii* (Fig. 1*H*), *C. brachystachya* (Fig. 1*F*), *C. cytisoides* and *C. depauperata*. Paracytic laterocyclic stomata with cells dividing in a variety of ways originating paracytic laterocyclic-like stomata with 1 + 3, 2 + 2, or 2 + 3 cells on each side of the stomata was observed in *C. blanchetii*, *C. brachystachya* (Fig. 1*F, G*), *C. cytisoides* and *C. decora* (Fig. 1*I*). Paracytic laterocyclic stomata with cells dividing at least twice, originating staurocytic-like stomata with 4–7 cells, were observed in *C. blanchetii* (Fig.1*H*) and *C. decora* (Fig. 1*I*). These stomata are still classified as paracytic laterocyclic because the cells originated from cell divisions giving raise to all these types of modified paracytic laterocyclic stomata do not show cell walls as thick as the other subsidiary cells which originated them; they persist as thin walled cells.

All eight species of *Chamaecrista* sect. *Absus* subsect. *Baseophyllum* displayed a single-layered epidermis (Fig. 2*A*–*F*). In *C. confertiformis*, *C. coriacea*, *C. depauperata* and *C. unijuga*, the epidermal cells of both leaflet blade sides had very thick outer periclinal walls. On the other hand, *C. blanchetii*, *C. brachystachya* (Fig. 2*F*) and *C. decora* showed thickenings only in the outer periclinal walls of the epidermal cells of the adaxial side. In opposition to all other species, thickenings on the epidermal cells were not observed in *C. cytisoides* (Fig. 2*G*). The thickenings observed in the epidermal cells were as high as or higher than the epidermal cells (Fig. 2*D*), sometimes three times higher than the epidermal cells. The height of these thickenings varied within samples of the same species. The epidermal cells near and at the leaflet margins were always smaller and had thicker cell walls than the other epidermal cells.

Leaflets mesophyll

Three of the eight species showed dorsiventral mesophyll (*C. cytisoides* (Fig. 2*G*), *C. decora* and *C. depauperata*). The palisade parenchyma of these species was made up of 3–4 layers of compact cells (Fig. 2*G*). The spongy parenchyma of *C. cytisoides* and *C. depauperata* was made up of 4–5 layers of loose cells (Fig. 2*G*). Conversely, a 6–7 layered spongy parenchyma with little space among the cells was present in *C. decora*. Isobilateral meshophyll was observed in the other five species (Fig. 2*A*–*C*). The palisade parenchyma of the adaxial side of *C. confertiformis* had three layers of compact cells (Fig. 2*A*); *C. blanchetii* had three layers; *C. brachystachya* 3–4 layers (Fig. 2*F*); and *C. coriacea* and *C. unijuga* (Fig. 2*B*), 4–5 layers. On the abaxial side, *C. blanchetii* and *C. brachystachya* (Fig. 2*F*) revealed to have a non-continuous

single-layered palisade parenchyma. Sometimes at same places the mesophyll even remembered a dorsiventral mesophyll (Fig. 2*F*). The palisade parenchyma on the abaxial side of *C. unijuga* was double-layered. In *C. confertiformis* (Fig. 2*A*) and *C. coriacea*, 3–4 layers were present.

All species had a hypodermis on the abaxial side. The number of cell layers in the hypodermis varied from 1–2 in *C. blanchetii* (Fig. 2*C, E*), *C. cytisoides* (Fig. 2*G*), *C. depauperata* and *C. unijuga*, to 1–3 in *C. brachystachya* (Fig. 2*F*), to 2–3 in *C. decora C. coriacea*. *C. confertiformis* was the only species to display a non-continuous single-layered hypodermis on the abaxial side (Fig. 2*A*). Mucilage idioblasts were observed in the palisade parenchyma and hypodermis in all species. *C. confertiformis* and *C. depauperata* also had mucilage idioblasts in the spongy parenchyma. *C. cytisoides* was the only species to have mucilage idioblasts typically found in the inner layers of the hypodermis (Fig. 2*G*). The number of layers of the hypodermis decrease with increasing the proximity to the leaf margins, being usually single-layered at the area. Mucilage substances could be found in the cellular spaces of the mesophyll cells of all species (Fig. 2*B, F*). *C. blanchetii* was the only species to reveal a layer of hypodermis with cells filled by phenolic compounds (Fig. 2*C, E*).

Collateral vascular bundles arranged in open arc were found in all species studied (Fig. 1*E, I,* 3*A*). The vascular bundles were surrounded by layers of fibres varying in number, from three to ten, depending on the size of the vascular bundle (Fig. 2*B, F*, 3*A*). A single-layered sheath of cells containing prismatic crystals (Fig. 3*A*) was observed surrounding the vascular bundles of all eight species studied. Tracheoids (enlarged tracheids) (Fig. 3*B*) were observed at the vein endings of all species studied.

Petiole/rachis

All petiole/rachis of all *Chamaecrista* studied had a single-layered epidermis (Fig. 3*C–E*) with few stomata. Hypodermis made up of 1–3 layers in the petiole/rachis was observed (Fig. 3*C–E*) in all species but *C. confertiformis* and *C. unijuga*. Chlorenchyma was observed in the petiolar cortex (Fig. 3*D*, *E*) of all species but *C. confertiformis* and *C. unijuga*.

In all species sampled but *C. blanchetii*, which has sessile leaves, the vascular tissue in the petiole/rachis formed a cylinder around a central pith of parenchymatic cells (Fig. 3*C–E*). The parenchyma cells of the petiole pith of these species showed thickened secondary walls (Fig. 3C, E). Two accessory bundles on the adaxial side, one at each side of the petiole/rachis, were found in most species (Fig. 3*C*). Sometimes 3–4 accessory bundles were observed (Fig. 3*D*). *C. decora* was the only species to typically display 4–6 accessory bundles (Fig. 3*E*). As for the vascular bundles in the leaflets, fibres surrounded the vascular system of the petiole (Fig. 3*A*) and the accessory bundles (Fig. 3*J*). A single-layered sheath of cells containing prismatic crystals was also observed surrounding the fibres (Fig. 3*A*, *F*).

Three types of petiole form and vascular system arrangement could be recognized on cross sections of the petiole/rachis. Type I (rounded petiole with typically two accessory bundles) was observed in *C. brachystachya*, *C. confertiformis*, *C. coriacea*, *C. cytisoides* (Fig. 3*C*) and *C. unijuga*). Type II (rounded petiole with two lateral lobes where two accessory bundles are typically found) in *C. coriacea*, *C. cytisoides* and *C. depauperata* (Fig. 3*D*). Type III (rounded petiole with 4–6 accessory bundles) was only found in *C. decora* (Fig. 3*E*).

Secretory structures and Trichomes

Extrafloral nectaries were observed on the petiole/rachis of all species as described in the Table 2.

Non-secretory trichomes were found at the leaflet base and scarcely spread on the adaxial side of the leaflet blades (Fig. 4*A*). Non-secretory trichomes were also found at the insertion of the petiole (pulvinus) on the stem and at the insertion of the petiolule (pulvinule) on the rachis of all species (Fig. 4*B*).

Non-vascularised colleters corresponding to the reduced type of the standard pattern at insertion of the petiole (pulvinus) on the stem and at the insertion of the petiolule (pulvinule) on the rachis (Fig. 4*B*) were observed in all species.

Mucilage idioblasts in the petiole/rachis as well as in both palisade and spongy parenchyma and hypodermis of the leaflets occurred as described in the other sections above.

Histochemical Tests

Histochemical tests carried out on the leaflets, revealed the presence of total polysaccharides (Fig. 4*C, D*), acid polysaccharides (Fig. 4*E*, *F*), acid muco-polysaccharides (Fig. 4*E, F*), pectins (Fig. 4*G*, *H*), mucilage (Fig. 4*I*) and phenolic compounds (Fig. 4*J, K*) in the mucilage idioblasts found in both palisade and spongy parenchymas as well as in the idioblasts found in the hypodermis of the leaflets. Besides having phenolic compounds within the mucilage idioblasts, *C. blanchetii* also had a layer of cells in the hypodermis which accumulated these substances. Mucilage idioblasts containing phenolic compounds revealed a curious pattern of

phenolic accumulation. Droplets of phenolic compounds apparently fused into larger drops which later filled all the cytoplasm (Fig. 4*J*, *K*). Lignins were only detected in the xylem cells and fibres. Sudan test turned negative results.

Phenetic Analysis

Principal component analysis (PCA) based on morphological and anatomical characters showed the clear separation of the eight species included within sect. *Absus* subsect. *Baseophyllum* (Fig. 6).

Identification key

An identification key for *Chamaecrista* sect. *Absus* subsect. *Baseophyllum* based only on anatomical characters was generated (below).

Identification key for *Chamaecrista* sect. *Absus* subsect. *Baseophyllum*.

1. Amphistomatic leaves……..…….……..……*C. confertiformis*
1'. Epistomatic leaves……..……2
    2. Dorsiventral mesophyll…….……...3
        3. Six- seven-layered spongy parenchyma……..…….…*C. decora*
        3'. Three- four- five-layered spongy parenchyma……....……4
            4. Mucilage idioblasts in the palisade and spongy parenchyma and hypodermis……..……...*C. depauperata*
            4'. Mucilage idioblasts typically forming a continuous layer of cell in the inner layer of the hypodermis…….……....…….*C. cytisoides*
    2'. Isobilateral mesophyll…….….5
        5. One non-continuous layer of palisade parenchyma in the adaxial side of the epidermis.....6
            6. Hypodermis with phenolic compounds…….....……...……..*C. blanchetii*
            6'. Hypodermis without phenolic compounds…….……..*C. brachystachya*
        5'. Two-three layers of palisade parenchyma in the abaxial side of the epidermis…...…...7
            7. Looped marginal ultimate venation……..……*C. coriacea*
            7'. Looped and incomplete marginal ultimate venation…….……...*C. unijuga*

Discussion

Anatomical characters and the Leguminosae

The single-layered epidermis observed in the species of *Chamaecrista* studied was also found in most Leguminosae (Solereder 1908; Metcalfe and Chalk 1950). Paracytic stomata type found in our study concurs to other reports in Cassiinae and Caesalpinioideae (Solereder 1908; Metcalfe and Chalk 1950; Cowan 1981; Saheed and Illoh 2010), being the amphistomatic leaves of *C. confertiformis* a singular anatomical trait for this species. As far as we are concerned, epistomatic leaves have not been reported in the Leguminosae family, which constitutes an apomorphy for the species of *Chamaecrista* studied. Amphistomatic leaves and anisocytic stomata are autapomorphies for *C. confertiformis*. The outlining of the anticlinal cell wall of the epidermal cells varies from straight to sinuous within the family Leguminosae (Lackey 1978; Rezende *et al*. 1994; Mendes and Paviani 1997; Luckow 2002); however the sinuous outlining of the anticlinal epidermal cell walls of *C. confertiformis* is useful to distinguish from the other *Chamaecrista* included within the subsect. *Baseophyllum*.

Dorsiventral and isobilateral mesophyll in our study were useful to distinguish the species included in the subsect. *Baseophyllum*.

Idioblasts varied their distribution among the *Chamaecrista* studied, being useful to distinguish the species. The mucilage idioblasts observed in the leaflet blade of the *Chamaecrista* studied may be the structures described by Lersten and Curtis (1996) as enlarged hypodermal cells in species of the tribe Caesalpinieae. Also, few species of *Caesalpinia* show enlarged subepidermal mucilage idioblasts in petals and sepals (Rudall *et al*. 1994). Idioblasts in the leaflets blades have also been described to other Leguminosae genera, *Caesalpinia* and *Parkinsonia* (Lersten and Curtis 1993; Lersten and Curtis 1994). The presence of the same contents (i.e. total

polysaccharides, acid polysaccharides, acid muco-polysaccharides, pectins, mucilage, and phenolic compounds) in all leaflet idioblasts studied makes the mucilage a unifying character for this subsection, as foliar idioblasts are of limited occurrence in the Caesalpinioideae (Lersten and Curtis 1993). In fact, Lersten and Curtis (1993) suggested that the pattern of distribution combined with identification of the contents of idioblasts cells could be useful in helping to determine taxonomic entities within the subfamily Caesalpinioideae.

Brochidodromous venation pattern with basal lateral veins, which would resemble the actinodromous-camptodromous-brochidodromous venation type (palmately veined leaves) of the *Chamaecrista* studied, have also been reported to other Leguminosae (Flores-Cruz 2004). However, the usual actinodromous-camptodromous-brochidodromous venation type found for the *Chamaecrista* species included within the subsect. *Baseophyllum*. is a synapomorphy for this subsection, constituting a unifying taxonomic character for this subsection.

Enlarged tracheids (tracheoids) at the vein endings and accessory bundles have also been reported for other Leguminosae genera were also (Solereder 1908; Metcalfe and Chalk 1950; Lersten and Curtis 1994, 1995, 1996; Francino *et al*. 2006; Pascal *et al*. 2000; Luckow 2002; Flores-Cruz 2004).

Oxalate crystals have also been reported for other Leguminosae species and are common for this family (Solereder 1908; Metcalfe and Chalk 1950; Cowan 1981; Watson 1981) and may represent a good taxonomic character for the family Leguminosae. Prismatic crystals were also reported in *Chamaecrista trichopoda* by Francino *et al.* (2006).

According to Thomas, colleters in Leguminosae are found only in the subfamilies Mimosoideae and Faboideae (1991). However, De-Paula and Oliveira (2007) reported the presence of colleters in the embryos of three *Chamaecrista* species, *C. desvauxii* var. *latistipula*, *C. flexuosa* and *C. nictitans* var. *patellaria*. Pascal et al. (2000) reported the presence of patches of glandular trichomes on the rachises of the tribes Caesalpinieae and Mimoseae. Such patches of glandular trichomes are actually very much structurally similar to colleters and could correspond to the colleters observed in our study. The presence of colleters in different genera of Leguminosae may indicate the promising use of these structures in taxonomic studies.

Although extrafloral nectaries are common in Leguminosae (Solereder 1908; Metcalfe and Chalk 1950), especially in the subfamily Mimosoideae, they are much less common in the subfamily Caesalpinioideae, being found mainly in the genera *Chamaecrista* and *Senna* (Lersten and Brubaker 1987; Pascal et al. 2000; Conceição et al. 2009). As these secretory structures are not supposed to be found in species belonging to the sect. *Absus*, they constitute a synapomorphy for the sect. *Absus* subsect. *Baseophyllum*.

Anatomical ecological adaptations of Chamaecrista *sect. Absus* subsect. Baseophyllum

Xeromorphic characters such as thickened outer periclinal cell walls, compact mesophyll, high amounts of vascularization and mechanical tissue (fibres), presence of mucilage idioblasts, and water storage hypodermis are indeed found in the species of *Chamaecrista* studied. Conceição (2006) states that six of the eight species

belonging to *Chamaecrista* sect. *Absus* subsect. *Baseophyllum* have coriaceous to semi-succulent leaves (i.e. *Chamaecrista blanchetii*, *C. brachystachya*, *C. confertiformis*, *C. coriacea*, *C. decora*, *C. unijuga*), an allusion to water store tissues, hypodermis, that are found in the leaves of these species.

The thickened outer epidermal walls displayed by the majority of species studied may play a role in UV-B filtration. The first line of defence in this regard is the epidermis, and thickened outer epidermal walls can reduce the penetration to more sensitive mesophyll tissues in the interior of the leaves (Day *et al*.; 1993, Rotondi *et al*. 2003). Moreover, according to Oertli *et al*. (1990), thick cell walls may help to prevent physical disruption of the cells through collapse during drying, and that may be the function played by the epidermal cells with thickened cell walls at the leaflet margins.

The number of palisade parenchyma layer is around 3–4 in the studied species and one may think that thickening a leaf may yield negative effects on carbon gain due to internal self-shading and increased resistance to CO_2 diffusion. However, thickening the leaf by adding more layers of palisade cells increases the carbon gains without greatly increasing the transpirational costs (Turner 1994), also strengthening (strength relating to the force to fracture the leaf) and toughening (relating to the energy and propagation of fracture) the leaf (Sanson et al. 2001). Moreover, in sunny open conditions, relative branching costs might be reduced by concentrating leaf mass into a smaller number of thicker leaves (Read and Stokes 2006; Read *et al*. 2009).

The presence of total polysaccharides, acid polysaccharides, acid muco-polysaccharides, pectins, and mucilage in the mucilage idioblasts found in both

palisade and spongy parenchymas as well as in the idioblasts found in the hypodermis of the leaflets may play a role in water retention because of their great ability to imbibe water (Fahn 1979; Fahn and Cutler 1992; Sawidis 1991; Christodoulakis *et al*. 1990; Al-Tardeh *et al*. 2009). Hygroscopic polysaccharides can bind 51 times their weight of water when hydrated in vivo (Nobel *et al*. 1992). Perhaps these mucilage idioblasts work in a similar way to a hydrenchyma. Christodoulakis *et al*. (1990) have also stated that mucilage cells can absorb water during the relatively humid season and conserve it for the vital activities of the leaf during the long arid seasons. Fahn (1979) also mentions that mucilage cells may serve as food reserve. Also, mucilage idioblasts could also work minimizing solar irradiation, aiding in the reduction of the temperature of the plant and blocking excess light, guaranteeing the optimal amount of light for the inner tissues (Christodoulakis *et al*. 1990; Voltolini *et al*. 2009).

The pattern of phenolic accumulation in the mucilage idioblasts containing phenolic compounds may be related to the phenolic compounds production. They seem to be produced and stored in small vesicles which later fuse and become a single large vesicle filled with phenolic compounds. Phenolic compound are thought to play a role in anti-herbivore defences by conferring unpalatability and toxicity to the plant organs where they are produced (Roshchina and Roshchina 1993) besides ensuring immunity of plants against bacterial or fungal infection (Roshchina and Roshchina 1993). Phenolic compounds can also maximize UV-B radiation-absorption, depleting UV-B radiation before it can reach the internal tissues (Nagel *et al*. 1998; Rotondi *et al*. 2003).

The enlarged tracheids (tracheoids) observed at the vein endings they may be involved in water storage as suggested by Luckow (2002).

The high amounts of fibres found around the vascular tissues may be associated with nutrient-poor soils (Loveless 1962; Beadle 1966; Turner 1994). Plants living in sunny areas where soils are poor in nutrient such as phosphorous and nitrogen are plenty of sunlight to perform photosynthesis, however nutrients are not readily available for growing, and therefore moderate nutrient deficiency limits growth more than photosynthesis (Herms and Mattson 1992). Carbohydrates accumulated in excess of growth requirements are then allocated to C-based secondary metabolites (Herms and Mattson 1992) such as lignins, which are a structural component of fibre walls.

The association of oxalate crystals with veins in leaves may be explained by the fact that Ca precipitation as crystalliferous solid inclusions in the cells surrounding the veins will prevent Ca accumulation around chlorenchyma cells, which could affect cellular function (Franceschi and Nakata 2005). Because sucrose transport in plants involves ATPase activity (Giaquinta 1979) and Ca^{2+} inhibits plasma membrane ATPase (Leonard and Hodges 1980), crystals may immobilize calcium where active sugar transport is at play (Vezza et al. 2006). Paiva and Machado (2005) found an increasing concentration gradient of calcium in the sieve elements from bundle sheath cells. As a result, the authors hypothesized that the formation of calcium oxalate crystals regulates calcium levels within the sieve elements, eliminating the excess of cytosolic calcium. Oxalate may also be involved in detoxification of hazardous metals such as aluminum, cadmium, cooper, lead, and strontium by incorporating these metals into the oxalate crystals (Franceschi and

Nakata 2005). *Chamaecrista* species are found in the "campos rupestres" and in the "cerrados" (Conceição 2006). These two biomes are known for having soils with high exchangeable aluminium levels and iron oxides (Lopes and Cox 1977; Benites *et al.* 2007; Vincent and Meguro 2008). Imprisoning heavy metals into oxalate crystals can be a strategy developed by the studied plants to cope with these elements which in high concentration may cause toxicity.

Colleters in the species studied may have a protective role, protecting tge apical meristem against desiccation by secreting viscous substances containing lipophilic substances or carbohydrate mucilage or both, as suggested by Thomas (1991) and Fahn (1979).

Anatomical characters as an additional tool to the taxonomy and phylogeny of the Chamaecrista *sect.* Absus *subsect.* Baseophyllum

Anatomical data have been used as a taxonomy tool supporting taxonomic studies not only for the family Leguminosae but also for many others (Solereder 1908; Metcalfe and Chalk 1950; Lackey 1978; Metcalfe and Chalk 1979; Crow *et al*. 1997; Luckow 2002, Flores-Cruz *et al*. 2004; Gomes *et al*. 2005; Araújo *et al*. 2010; Saheed and Illoh 2010).

Although with minor exception for one or other species, the presence of epistomatic leaves, paracytic laterocyclic stomata and all other modifications of this type of stomata, mucilage idioblasts, collateral vascular bundles, 2–6 accessory bundles, a sheath of oxalate crystals around vascular bundles, layers of fibres around the vascular bundles, extrafloral nectaries, non-secretory trichomes at the base of adaxial site of the pulvinus and pulvinule, colleters, and actinodromous-

camptodromous-brochidodromous (palmately veined leaves) are considered unifying taxonomic characters for the species included within the subsect. *Baseophyllum*.

Anatomical characters such as the type and placement of stomata on leaves, type of mesophyll, presence/absence of mucilage idioblasts in the palisade and spongy parenchymas and hypodermis, presence/absence of phenolic compounds in the hypodermis and marginal ultimate venation produced a short concise informative identification key based only on anatomical characters for the eight species found within the subsect. *Baseophyllum*. showing to be promising anatomical and morphological characters useful for the taxonomy of this subsection.

Moreover, as the eight species could be clearly separated from each other by using either the anatomical characters only, as shown in the identification key, or both morphological and anatomical characters, as shown in the PCA graph, our results corroborate the taxonomic re-arrangement proposed by Conceição *et al*. (2008), which elevates the varieties included in *Chamaecrista cytisoides* at the species rank.

Conclusion

Anatomical and morphological characters showed to be useful for unifying the species included in the subsect. *Baseophyllum* as monophyletic subsection as a monophyletic group. They were also important in the separation of the *Baseophyllum* species as defined by the molecular studies, corroborating the taxonomic re-arrangement proposed by Conceição *et al*. (2008).

Anatomical characters alone were also useful for elaborating a dichotomous anatomical key for the identification of the species. Moreover, the anatomical

characters displayed by the species included in the subsect. *Baseophyllum* seem to be anatomical adaptations which allow the survival of these species at the harsh environmental where they are found.

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Table 1. Material collected from the herbaria of the Universidade Estadual de Feira de Santana (HUEFS) and Universidade de São Paulo (SPF) and fresh materials recollected in the field according to coordinates given by Conceição et al. (2008).

Irwin and Barneby (1982)	**Conceição *et al.* (2008)**	**Herbaria**	**Fresh Material Recollected in the Field**	**Vegetation type where the species are found**
C. coriacea	*C. coriacea*	HUEFS 112.739 SPF 179.573	Yes	campo rupestre
-----	*C. depauperata* Conceição *et al.* (2001)	HUEFS 94.669 HUEFS 112.632 SPF 179.580	No	campo rupestre
C. cytisoides var. *blanchetii*	*C. blanchetii*	HUEFS 66.389 HUEFS 131.380 SPF 90.113	Yes	campo rupestre, cerrado, restinga
C. cytisoides var. *brachystachya* and *C. cytisoides* var. *micrantha*	*C. brachystachya*	HUEFS 12.469 HUEFS 17.060 SPF 179.567	Yes	caatinga,campo rupestre, cerrado, restinga
C. cytisoides var. *confertiformis*	*C. confertiformis*	HUEFS 65.042 HUEFS 65.038 SPF 179.572	No	campo rupestre
C. cytisoides var. *cytisoides*	*C. cytisoides*	HUEFS 105.351 HUEFS 71.567 SPF 160.397	Yes	transition between campo rupestre and fog forest (cloud forest)
C. cytisoides var.	*C. decora*	HUEFS 75.078 SPF 42.876	Yes	campo rupestre
C. cytisoides var. *unijuga*	*C. unijuga*	HUEFS 101.773 HUEFS 73.833	No	restinga

Table 2. *Chamaecrista* species and respective gland position, number, and shape.

Species	Position	Number	Shape
C. blanchetii (Fig. 1*A*)	Between the single pair of leaflets	1	Rounded/elliptical concave or flat triangular shaped
C. cytisoides	On the interfoliolar segments of the rachis	1–2	Rounded/elliptical concave
C. coriacea	Near the middle of the petiole	1	Rounded/elliptical concave
C. decora (Fig. 1*B*)	Mostly near the middle of the petiole. Few glands are place below the upper pair of leaflets	1	Rounded/elliptical concave
C. brachystachya	On the interfoliolar segments of the rachis or between the lower pair of leaflets	1–2	Rounded/elliptical concave
C. confertiformis	Below the upper pair of leaflets	1	Rounded/elliptical concave
C. depauperata	Middle of the petiole	1	Rounded/elliptical concave
C. unijuga	Middle of the petiole	1	Rounded/elliptical concave

Fig 1. Diaphanisations of leaflet blades of *Chamaecrista* species. (*A*) *C. depauperata* showing the actinodromous venation type. (*B*, *C*) Camptodromous-brochidodromous marginal ultimate venation. (*B*) *C. coriacea*, typical looped marginal ultimate venation. (*C*) *C. depauperata*, incomplete marginal ultimate venation. (*D*) *C. confertiformis*, anisocytic stomata. (*E*) *C. coriacea*. (*F*, *G*) *C. brachystachya*. (*H*) *C. blanchetii*. (*I*) *C. decora*. (*J–L*) *C. cytisoides*. Subsidiary cells of the paracytic laterocyclic stomata dividing in a variety of planes giving rise to different types of paracytic laterocyclic staurocytic-like stomata. Asterisk, paracytic laterocyclic with 1 + 2 subsidiary cells; black arrow, paracytic laterocyclic staurocytic-like stomata with varying numbers subsidiary cells; double black arrows indicate division in the subsidiary cells; double white arrow, paracytic laterocyclic anisocytic-like stomata; white arrow, paracytic laterocyclic stomata.

Fig 2. Anatomy of leaflet blades of *Chamaecrista* species from herborized materials. (*A–E*) Cross sections. (*A*) *C. confertiformis*. (*B*) *C. unijuga*. (*C–E*) *C. blanchetii*. (*D*) Detail of the thickened outer periclinal walls of the epidermis on the adaxial side. (*E*) Detail of the double-layered hypodermis with phenolic compounds on the abaxial side. (*F*) *C. brachystachya*. Note the enlarged tracheids (tracheoids). (*G*) *C. cytisoides*. Arrow, tracheoids; asterisk, mucilage idioblasts.

Fig. 3. (A, B) Leaflets of *Chamaecrista* species on (*A*) cross sections and (*B*) paradermal section. (*A*) *C. unijuga* showing the vascular bundle and (*B*) *C. decora* showing the enlarged tracheids (tracheoids). (*C–E*) Cross and (*F*) longitudinal sections of the (*C*) rachis and (*D–F*) petiole. (*C*) *C. cytisoides*. (*D*, *F*) *C*

.*depauperata*. (*F*) *C. decora*. Cc, cortex with chlorophyll; Fi, fibres; Tr, tracheiods; Ph, phloem; Xy xylem, Sc, sheath of crystal; arrow, cortex with chlorophyll; double arrow, hypodermis;

Fig. 4. Leaflets of *Chamaecrista* species. (*A*, *B*) Diaphanizations (*C–K*) Cross sections. (*A*, *B*) *C. cytisoides* showing (*A*) non-secretory trichomes on the adaxial side at the leaflet base and (*B*) colleters and trichomes at the adaxial side of the petiolule/pulvinule. (*C–J*) Positive histochemical tests results. Note the reactions with the mucilage idioblasts. (*C*, *D*) Schiff Periodic Acid, *C. decora*. Note the mucilage idioblasts in (*C*) the palisade parenchyma and (*D*) hypodermis. (*E*, *F*) Alcian blue. (*E*) *C. coriacea*. Note the mucilage idioblasts in the palisade parenchyma and hypodermis. (*F*) *C. decora*. Palisade parenchyma. (*G*, *H*) Ruthenium red. (*G*) *C. blanchetii*. Note the mucilage idioblasts in the hypodermis. (*H*) *C. decora*. Palisade parenchyma. (*I*) Tannic acid, *C. blanchetii*. Hypodermis. (*J*, *K*) Ferrous sulphate in formalin buffer, *C. coriacea*. Palisade parenchyma. From left to right, note that the droplets of phenolic compounds seem to fuse. (*K*) Detail of the vesicles containing phenolic compounds. Arrow, trichome; double arrow, colleter; asterisk, mucilage idioblasts.

Fig. 5. Principal component analysis (PCA of the eight species included within sect. *Absus* subsect. *Baseophyllum* showing the clear separation among the species.

Appendix 1. Anatomical characters of *Chamaecrista* section *Absus* subsection *Baseophyllum* used in the phenetic analyses.

char 1. Very thickened periclinal external wall on both epidermal sides (cell wall height as high as or higher than the epidermal cell)
char 2. Very thickened periclinal external wall on the adaxial side and little thickened on the abaxial side (cell wall height smaller than half of the epidermal cell height).
char 3. Straight/tabular outlining of the anticlinal epidermal cell wall on both epidermal sides.
char 4. Waved/sinuous outlining of the anticlinal epidermal cell wall on both epidermal sides.
char 5. Epistomatic leaves.
char 6. Amphistomatic leaves.
char 7. Anisocytic stomata.
char 8. Paracytic laterocyclic stomata (1 + 1 cell on each side of the stomata).
char 9. Paracytic laterocyclic stomata (1 + 1 cell on each side of the stomata, one of the two cells divides anticlinally in relation to the guard cell originating anisocytic-like stomata.
char 10. Paracytic laterocyclic stomata (1 + 1 cells on each side of the stomata, but cells divide originating 1 + 3 or 2 + 2 or 2 + 3 paracytic laterocyclic-like stomata, as only 2 cells are in touch with the guard cell).
char 11. Paracytic laterocyclic stomata (1 + 1 cells on each side of the stomata, but cells divide originating staurocytic-like stomata with 4–7 cells).
char 12. Non-continuous single-layered hypodermis on the leaf blade present.
char 13. Typically double-layered hypodermis.
char 14. Hypodermis with phenolic compounds.
char 15. Isobilateral mesophyll.
char 16. Dorsiventral mesophyll.
char 17. Three-layered palisade parenchyma on the adaxial side.
char 18. Three/four-layered palisade parenchyma on the adaxial side.
char 19. Four/five-layered palisade parenchyma on the adaxial side.
char 20. Non-continuous single-layered palisade parenchyma on the abaxial side.
char 21. Two/three-layered palisade parenchyma on the abaxial side.
char 22. Three- four- or five-layered spongy parenchyma.
char 23. Six/seven layered spongy parenchyma.
char 24. Mucilage idioblasts present only in the palisade parenchyma and hypodermis.
char 25. Mucilage idioblasts in the palisade and spongy parenchyma and hypodermis present.
char 26. Mucilage idioblasts typically found in the inner layers of the hypodermis.
char 27. Looped marginal ultimate venation.
char 28. Looped and incomplete marginal ultimate venation.
char 29. Incomplete marginal ultimate venation.

char 30. Sessile leaves.
char 31. Petiole with 2–5 mm present.
char 32. Petiole with more than 5 mm present.
char 33. Rachis typically present.
char 34. Chlorophyll found in the cortex of the petiole.
char 35. Cortex of the petiole without chlorophyll.
char 36. Vascular system of the petiole Type I (rounded petiole with typically two accessory bundles).
char 37. Vascular system of the petiole Type II (rounded petiole with two lateral lobes where two accessory bundles are typically found).
char 38. Vascular system of the petiole Type III (rounded petiole with 4–6 accessory bundles).
char 39. Hypodermis in the petiole present.
char 40. Extrafloral nectaries usually near the middle of the petiole.
char 41. Extrafloral nectaries between the single pair of leaflets.
char 42. Extrafloral nectaries on the interfoliolar segment of the rachis.
char 43. Extrafloral nectaries between the lower pair of leaflets.
char 44. Extrafloral nectaries below the upper pair of leaflets
char 45. Extrafloral nectaries rounded concave/elliptical concave only.
char 46. Extrafloral nectaries rounded concave/elliptical concave and flat-triangular shaped.
char 47. One pair of leaflets, rarely two.
char 48. Two pairs of leaflets.
char 49. Three or more pairs of leaflets.
char 50. Subshrub procumbent or prostate.
char 51. Shrubs or small trees.
char 52. Underground xylopodium.
char 53. Reddish or purplish petiole
char 54. Green petiole.

Appendix 2. Matrix with species *vs.* anatomical and morphological characters created in the Microsoft Excel program Version 11.0. (1) indicates the presence and (0) the absence of the character.

	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q
	char1	char2	char3	char4	char5	char6	char7	char8	char9	char10	char11	char12	char13	char14
blanc	0	1	1	0	1	0	0	0	1	1	1	0	1	1
brach	0	1	1	0	1	0	0	1	1	1	0	0	1	0
confe	1	0	0	1	0	1	1	1	0	0	0	1	0	0
coria	1	0	1	0	1	0	0	1	0	0	0	0	1	0
cyti	0	0	1	0	1	0	0	0	1	1	0	0	1	0
deco	0	1	1	0	1	0	0	0	0	1	1	0	1	0
depa	1	0	1	0	1	0	0	1	1	0	0	0	1	0
unij	1	0	1	0	1	0	0	1	0	0	0	0	1	0

	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q
	char15	char16	char17	char18	char19	char20	char21	char22	char23	char24	char25	char26	char27	char28
blanc	1	0	1	0	0	1	0	1	0	1	0	0	0	1
brach	1	0	0	1	0	1	0	1	0	1	0	0	0	1
confe	1	0	1	0	0	0	1	1	0	0	1	0	0	1
coria	1	0	0	0	1	0	1	1	0	1	0	0	1	0
cyti	0	1	0	1	0	0	0	1	0	1	0	1	0	1
deco	0	1	0	1	0	0	0	0	1	1	0	0	0	1
depa	0	1	0	1	0	0	0	1	0	0	1	0	0	0
unij	1	0	0	0	1	0	1	1	0	1	0	0	0	1

	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q
	char29	char30	char31	char32	char33	char34	char35	char36	char37	char38	char39	char40	char41	char42
blanc	0	1	0	0	0	0	0	0	0	0	0	0	1	0
brach	0	0	1	0	1	1	0	1	0	0	1	0	0	1
confe	0	0	1	0	1	0	1	1	0	0	0	0	0	0
coria	0	0	0	1	1	1	0	1	1	0	1	1	0	0
cyti	0	0	1	0	1	1	0	1	1	0	1	0	0	1
deco	0	0	0	1	0	1	0	0	0	1	1	1	0	0
depa	1	0	0	1	0	1	0	0	1	0	1	1	0	0
unij	0	0	0	1	0	0	1	1	0	0	0	1	0	0

	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q
	char43	char44	char45	char46	char47	char48	char49	char 50	char 51	char52	char53	char54
blanc	0	0	0	1	1	0	0	0	1	0	0	1
brach	1	0	1	0	0	1	0	0	1	0	0	1
confe	0	1	1	0	0	1	0	0	1	0	0	1
coria	0	0	1	0	0	1	0	1	0	1	0	1
cyti	0	0	1	0	0	0	1	0	1	0	0	1
deco	0	0	1	0	1	0	0	0	1	0	1	0
depa	0	0	1	0	1	0	0	1	0	1	1	0
unij	0	0	1	0	1	0	0	0	1	0	0	1

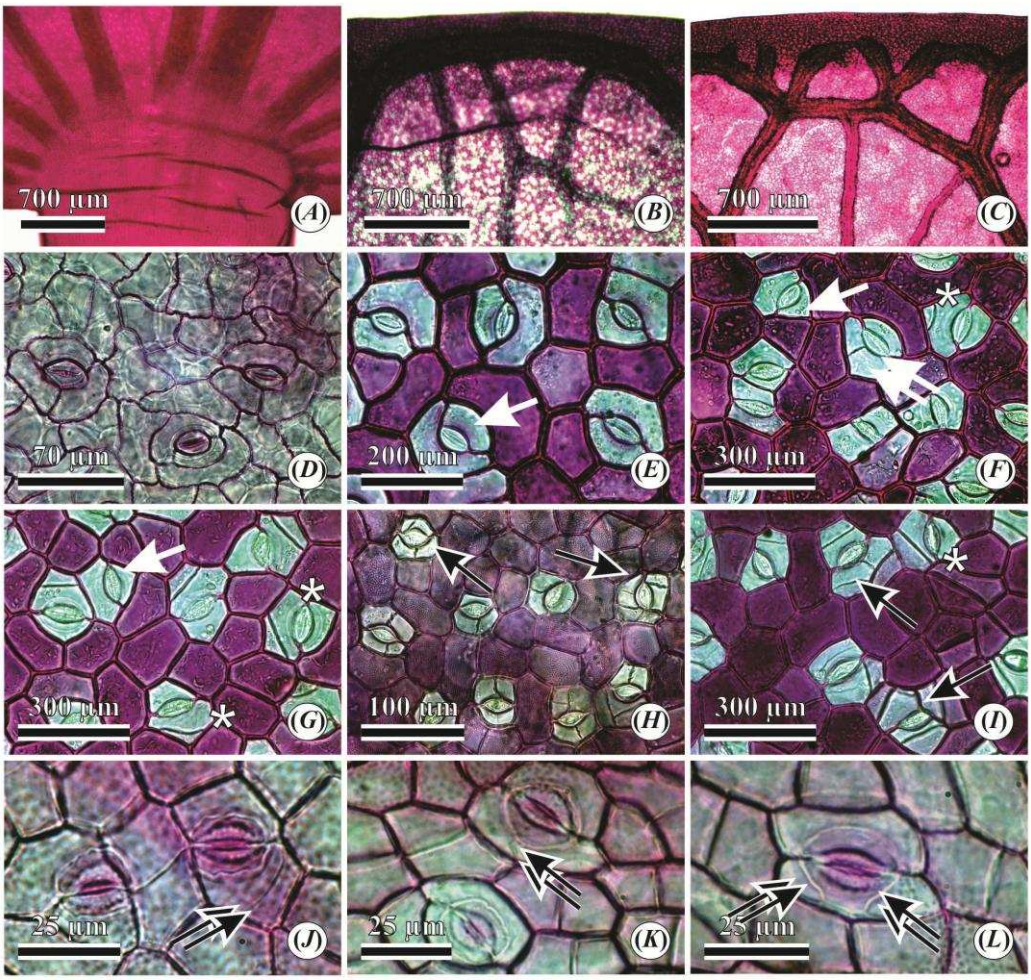
700 µm
(A)
700 µm
(B)
700 µm
(C)
70 µm
(D)
200 µm
(E)
300 µm
(F)
300 µm
(G)
100 µm
(H)
300 µm
(I)
25 µm
(J)
25 µm
(K)
25 µm
(L)

Figure 1

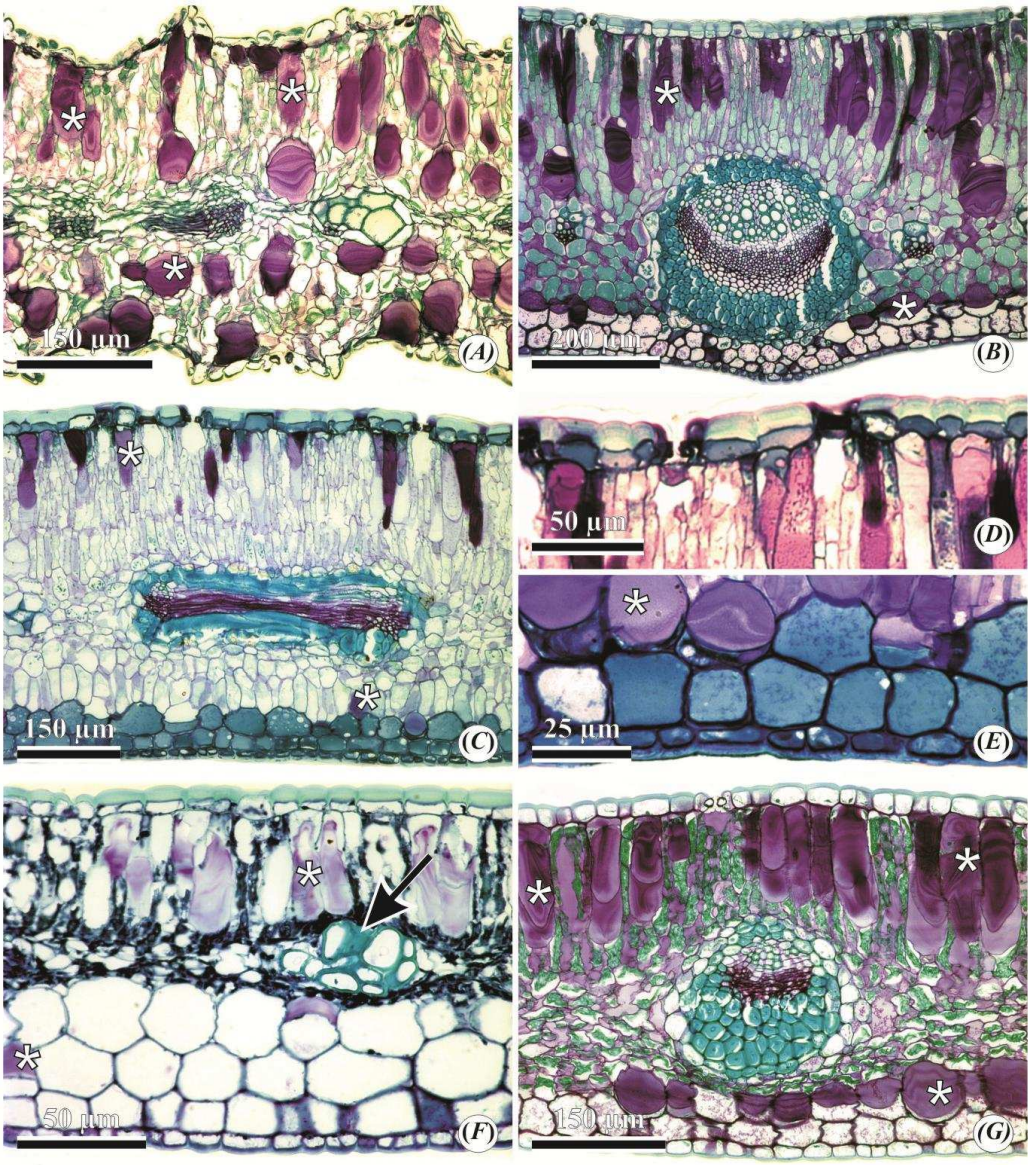

Figure 2

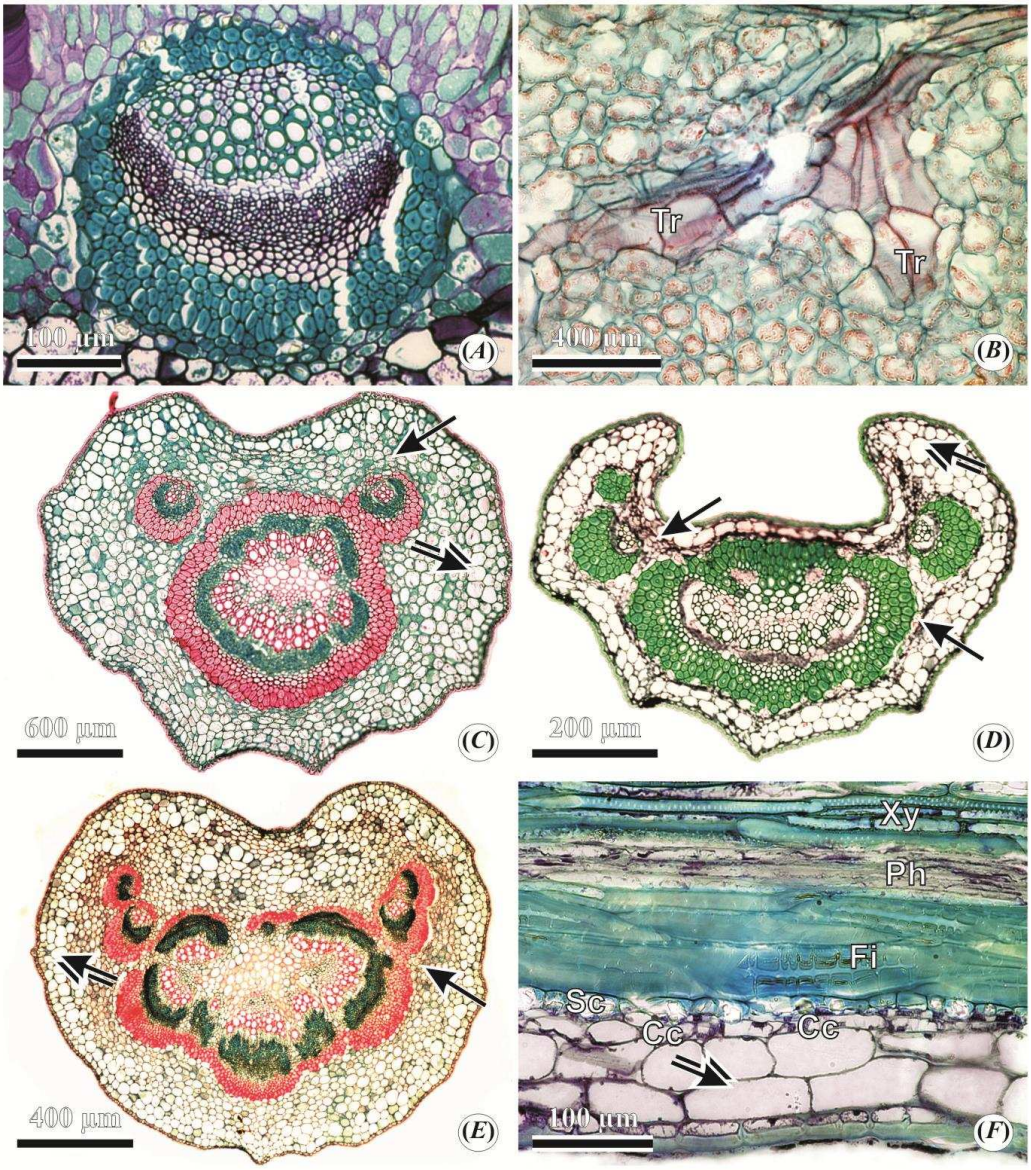
100 µm
(A)
Tr
Tr
400 µm
(B)
600 µm
(C)
200 µm
(D)
400 µm
(E)
Xy
Ph
Fi
Sc
Cc
Cc
100 µm
(F)

Figure 3

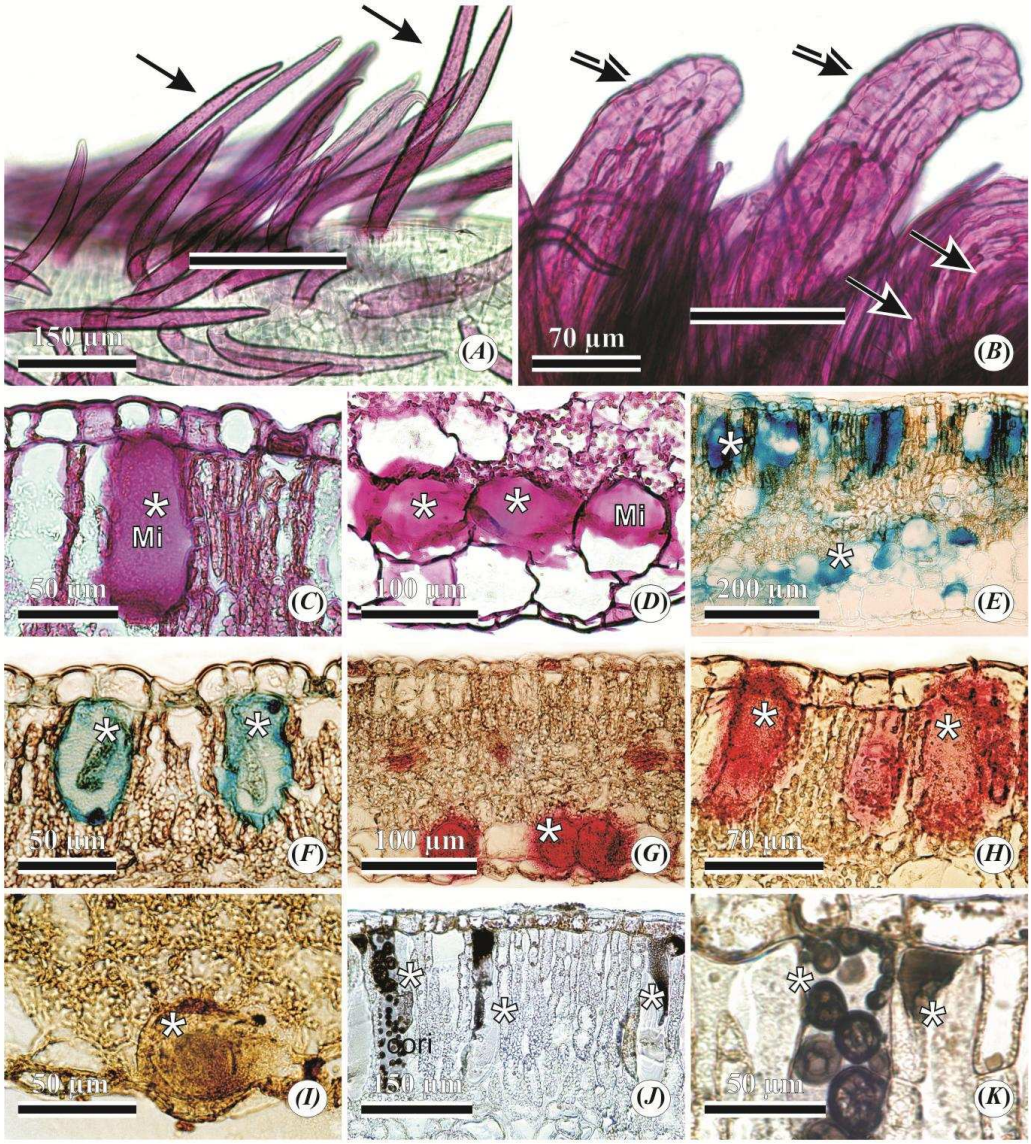
150 µm
(A)
70 µm
(B)
Mi
50 µm
(C)
Mi
100 µm
(D)
200 µm
(E)
50 µm
(F)
100 µm
(G)
70 µm
(H)
50 µm
(I)
ori
150 µm
(J)
50 µm
(K)

Figure 4

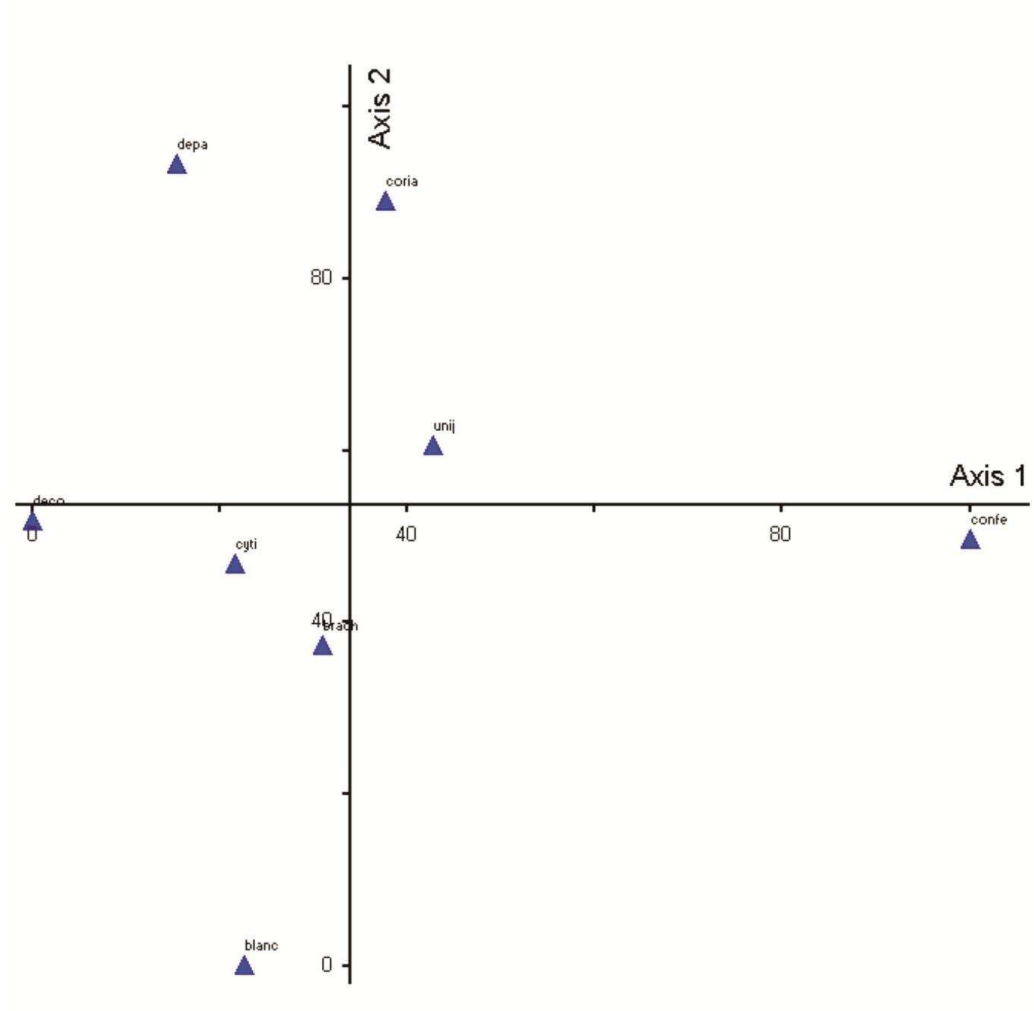
Axis 2
Axis 1
depa
coria
unij
deco
cyti
brach
blanc
confe
80
40
0
80
40
0

Figure 6

5. CONCLUSÕES GERAIS

A posição a morfologia externa, a anatomia, a detecção de glicose na seção, assim como polissacarídeos e outras substâncias nos permitiu caracterizar as glândulas encontradas no pecíolo/raque das espécies de *Chamaecrista* seção *Absus* subseção *Baseophyllum* como nectários extraflorais (NEF).

Embora todos os NEFs compartilhem caracteres morfo-anatômicos que não são úteis o bastante para distinguir uma espécie da outra, esta similaridade morfo-anatômica ainda sim possui um valor taxonômico promissor, unindo essas espécies num único grupo, dando suporte a monofilia desta subseção.

Além dos NEFs, outros caracteres morfo-anatômicos dos folíolos e pecíolo/raque mostraram-se úteis, unificando as espécies circunscritas na subseção *Baseophyllum*, o que também corrobora com a monofilia desta subseção. Caracteres morfo-anatômicos também foram úteis na distinção das variedades de *Chamaecrista cytisoides*, corroborando com dados moleculares que sugerem a elevação dessas variedades ao nível de espécies.