Flora 207 (2012) 427–435

Contents lists available at SciVerse ScienceDirect

Flora

journal homepage: www.elsevier.de/flora

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

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ARTICLE INFO

Article history:
Received 7 August 2011
Accepted 1 March 2012

Keywords:
"campo rupestre"
Extrafloral nectary
Histochemistry
Mucilage idioblast
Ontogenesis
Cassiinae

ABSTRACT

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

Introduction

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

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

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

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

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

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

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0367-2530/$ – see front matter © 2012 Published by Elsevier GmbH.
http://dx.doi.org/10.1016/j.flora.2012.03.007

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

Materials and methods

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

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

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

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

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

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

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

Results

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

Gland ontogenesis

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

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

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

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

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