

# Morphology, ontogeny and structure of the stipular nectaries in *Caamembeca spectabilis* (Polygalaceae)

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## ABSTRACT

Nodal glands are found in one third of the Polygalaceae genera and have valuable taxonomic, ecological and evolutionary significance. In Brazil, they occur in five of the eleven genera already registered. However, there is still a controversy regarding the origin of these structures. The objective of this study was to characterize the morphology and the origin of nodal glands in *Caamembeca spectabilis*, in order to increase the structural and functional knowledge of these glands in the genera. Samples of nodal regions were collected, fixed and processed according to the methods of light microscopy and electron scanning. Ants were observed and identified along the stem axis. The glucose in exudate allows us to classify these glands as extrafloral nectaries. They are located in pairs on the nodal region. However, its origin is in the leaf trace. In the longitudinal section, the nectaries were present in the apex of cells with anticlinal walls impregnated with suberin, which represents the first record for the family. In this region there is also the formation of a hole by lysis. The secretory tissue is surrounded by phloem. Xylem vessels were observed only on the basis of the nectary, where there are also idioblasts with crystals in druse type. We have studied the ontogeny of the glands nodal in *Caamembeca spectabilis* and unveiled that these glands are linked to the leaves as stipular nectaries. In addition, the new findings presented here may add support for the understanding of morphology and anatomy of nodal glands in *Caamembeca*.

**KEYWORDS:** Fabales, extrafloral nectaries, vascularization, suberin, ants.

## Morfologia, ontogenia e estrutura dos nectários estipulares em *Caamembeca spectabilis* (Polygalaceae)

### RESUMO

Glândulas nodais são encontradas em um terço dos gêneros de Polygalaceae e possuem grande importância taxonômica, ecológica e evolutiva. No Brasil, ocorrem em cinco, dos onze gêneros já registrados. Contudo, ainda há controvérsias quanto à origem dessas estruturas. O objetivo deste trabalho foi caracterizar a morfologia e a origem das glândulas nodais em *Caamembeca spectabilis*, visando ampliar o conhecimento estrutural e funcional dessas glândulas no gênero. Amostras das regiões nodais foram coletadas, fixadas e processadas segundo os métodos em microscopia de luz e eletrônica de varredura. Formigas foram observadas e identificadas ao longo do eixo caulinar. A glicose no exsudato permite tratar as glândulas como nectários extraflorais. Esses estão localizados aos pares na região nodal. Porém, sua origem ocorre no traço foliar. Os nectários, em secção longitudinal, apresentam as células do ápice com paredes anticlinalis impregnadas com suberina, sendo este o primeiro registro para a família. Nessa região também ocorre a formação de um orifício por lise. O tecido secretor do nectário é circundado pelo floema. Vasos de xilema foram observados apenas na base do nectário, onde também ocorrem idioblastos com cristais do tipo drusas. Dessa forma, este estudo confirma a trajetória ontogenética das glândulas nodais em *C. spectabilis*, as quais, de fato, estão ligadas às folhas, como nectários estipulares. Além de apresentar dados inéditos que auxiliam a compreensão morfológica e anatômica das mesmas em *Caamembeca*.

**PALAVRAS-CHAVE:** Fabales, nectários extraflorais, vascularização, suberina, formigas.

## INTRODUCTION

The Polygalaceae comprises 20 genera with 1.200 species distributed in tropical and temperate regions, especially in the Americas (Paiva 1998, Souza and Lorenzi 2012). In Brazil this family is represented by eleven genera and 200 species (Pastore *et al.* 2015), which occur in all vegetation formations (Aguiar-Dias *et al.* 2012).

The secretory structures have an important generic and specific contribution to the family, but knowledge on them is scarce and, often they are only referred to as present or absent (Aguiar and Aranha Filho 2008, Coelho *et al.* 2008, Lüdtke and Aguiar 2008, Pastore and Marques 2009). Among these structures are the nodal extrafloral nectaries (EFNs) which are specialized glands or tissues that secrete nectar and are found on the vegetative portions of many plant species, usually functioning as a defensive mechanism against herbivores (Fahn 1979, Barônio 2012).

The nodal glands are found in about one third of the Polygalaceae genera, occurring in five (*Caamembeca*, *Diclidanthera*, *Monnina*, *Moutabea* and *Securidaca*) out of the eleven genera registered in Brazil (Pastore *et al.* 2015). In *Caamembeca* and one of the morphologic structures that delimitate the genus are the nodal glands (Pastore 2012). There is a controversy about the origins of these structures where some researches considered them to be leaf structures (Chodat 1891), while other studies regarded them as stem structures (Eriksen and Persson 2007, Marques and Peixoto 2007).

Recent anatomical studies found that nodal nectaries of *Caamembeca laureola* (cited as *Polygala laureola*) are connected to the leaf trace, classifying them as stipular nectaries (Aguiar-Dias *et al.* 2011). However studies with the nodal ENFs of *Caamembeca* are clearly relevant to unravel whether this structure is shared among all species.

*Caamembeca spectabilis* is an Amazon native species used as a medicinal resource by the local people of the region, for the treatment of diverse diseases such as diarrhea, diabetes, dysentery, vaginal discharge and hemorrhoid (Coelho-Ferreira 2009), but can also have an ecological and taxonomic importance, due to the presence of nodal glands. The objective of this research was to characterize the morphology and ontogeny of nodal glands in *C. spectabilis*.

## MATERIALS AND METHODS

Samples of *Caamembeca spectabilis* (DC.) J.F.B.Pastore were collected at Museu Paraense Emílio Goeldi, Belém, PA (1°27'5,69"S and 48°26'40,38"W). One voucher was deposited in the João Murça Pires Herbarium under number MG199399.

Field observations were made during the daylight hours (08:00 – 18:00 h) for five consecutive days in August and December of 2012. In the field, glucostrips (Glicofita Plus®, Accu-Chek Active® - F. Hoffmann La Roche Ltd.©) were used to test for the occurrence of glucose in the exudate. Insects visiting the glands to collect the exudate were preserved in 70% ethanol and identified.

Samples of nodal nectaries were fixed in FAA (formalin, acetic acid, 50% alcohol) for 24 h (Johansen 1940). The material was isolated and dehydrated in a graded tertiary butanol series and embedded in paraffin (Paraplast Plus®, Sigma-Aldrich, Germany) (Johansen 1940). Cross and longitudinal sections (10 - 15 µm thickness) were obtained with a rotary microtome (Leica RM 2245, Nussloch, Germany) and stained with safranin and astra blue (Gerlach 1969). Permanent glass slides were mounted in synthetic resin (Permount, New Jersey, USA). The samples were examined under polarized light in order to better document birefringent cell constituents. The photomicrographs were obtained with a photographic microscope (Axiolab, Zeiss, Jena, Germany) with a coupled Canon digital camera.

The nodal samples were fixed in FAA (formalin, acetic acid, 50% alcohol) for 24 h and dehydrated in an ethanol series (Johansen 1940). Subsequently, samples were dried at critical point and sputtered with a thin layer of gold (Robards 1978) and examined with a scanning electron microscope (Leo Electron Microscopy Ltd, 1450 VP, Cambridge, England).

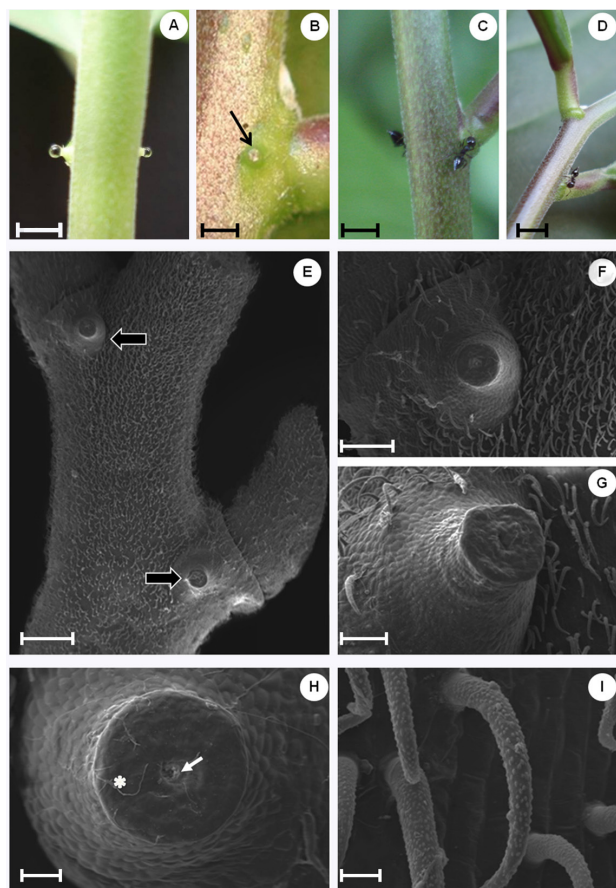
The following histochemical tests were applied on cross section of nodal glands: Sudan III to suberin (Sass 1951), fluoroglucinol to lignin (Johansen 1940) and hydrochloric acid and acetic acid to calcium oxalate crystals (Chamberlain 1932). To describe nectaries we used the morphological terms available elsewhere (Marques and Peixoto 2007; Aguiar-Dias *et al.* 2011).

## RESULTS

### Stipulates EFNs morphology and field observations

The extrafloral nectaries (EFNs) of *Caamembeca spectabilis* were observed near the nodal region in pairs on the lateral base of petiole (Figures 1A-D). The exudate was abundant (Figure 1A). *Crematogaster* ants were observed collecting the nectar from the nodal EFNs (Figures 1C-D).

At maturity, the nodal EFNs have a cylindrical shape, approximately 0.7 mm in length and 0.5 mm in width (Figures 1E-H) with some trichomes on the glands base (Figures 1F-G, 1I). The apical region of EFNs has a central orifice that releases nectar (Figures 1G-H). Fungal hyphae were observed on the glands surface, particularly near the central orifice (Figure 1G).

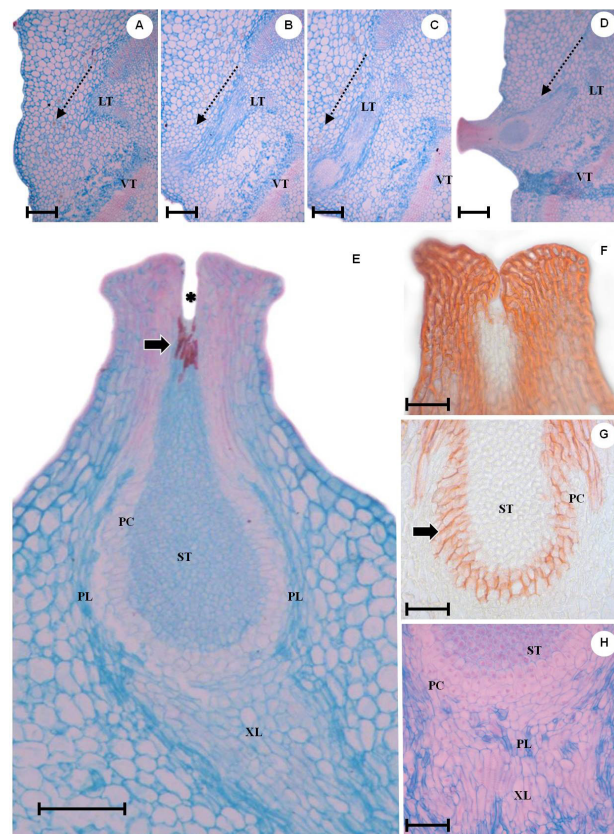


**Figure 1.** Field observations and stipulates EFNs morphology of *Caamembecka spectabilis*. A. EFNs exudate. B. Position of gland (arrow). C-D. *Crematogaster* ants on the EFNs. E. Nodal position of glands (arrows). F-G. Detail of glands. H. Detail of apex, central orifice (arrow) and fungal hyphae (asterisk). I. Ornamentation of nodal trichomes. Scale bars: A, C-D: 5 mm; B: 2mm; E: 700  $\mu$ m; F: 250  $\mu$ m; G: 120  $\mu$ m; H: 60  $\mu$ m; I: 15  $\mu$ m. "This figure is colored in the electronic version"

### Stipulates EFNs ontogeny and structure

The EFNs have vascular origin at the leaf trace, therefore each leaf is connected to a pair of EFNs (Figures 2A-D). In the nodal region preceding the nectary there is a vascular cylinder that will result a unilacunar foliar trace emitting from its extremities, and two opposite collateral bundles to the base of each nodal EFNs (Figures 2A-D). Past the nodal region, the vascular cylinder returns to whole form again.

The EFNs in secretory phase showed, in longitudinal sections, uniseriate epidermis (Figures 2E, 3A-D). The periclinal wall of epidermal cells has a thin cuticle throughout its length; at its apical center a possible orifice formed by lysis process was observed, where the nectar is secreted (Figures 3A-C).



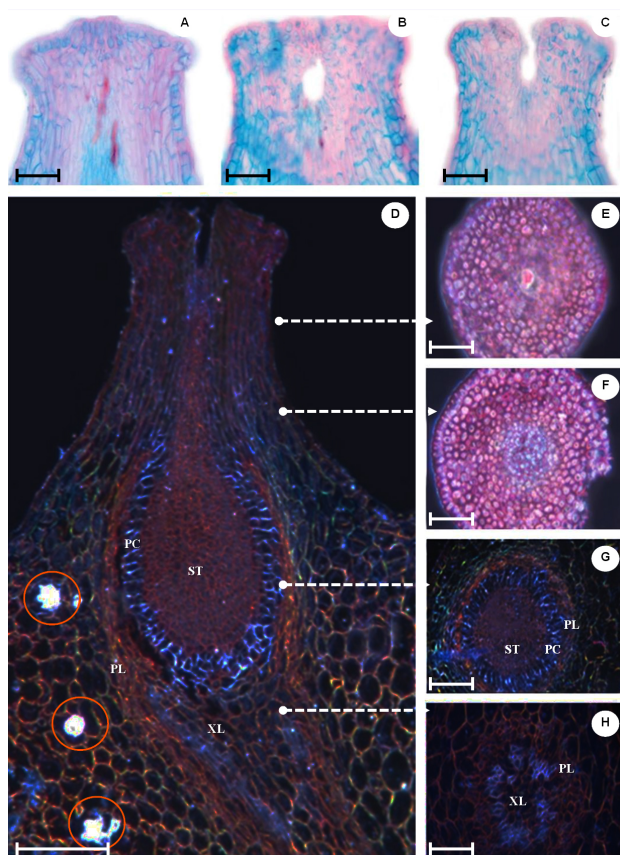
**Figure 2.** Stipulates EFNs ontogeny and structure of *Caamembecka spectabilis*. A-D: EFNs vascularization. A-C. Nodal region, in cross section. D. Glands connect to the leaf trace (arrow). E. EFNs in the longitudinal section, secretion (arrow), central orifice (asterisk). F. Detail of apical cells with suberin encrusted. G. Detail of polygonal cells (arrow). H. Basal region with the vascularization formed by xylem and phloem and the cells of secretory tissue (EFN: Extrafloral nectaries. LT: leaf trace, VT: Vascular trace, ST: Secretory tissue, PC: Polygonal cells, XL: Xylem, PL: Phloem) Scale bars: A- D: 150  $\mu$ m; E: 80  $\mu$ m; F-H: 50  $\mu$ m. "This figure is colored in the electronic version"

The parenchyma cells in the cortical position had periclinal and anticlinal walls with suberin encrusted, especially in the cell layer closer to the apex of glands (Figure 2F). The secretory tissue occupied the medullar region (Figures 2E, 3D). Its cells are small, thin-walled and have an evident nucleus (Figures 2E, 2H).

In cross section, the nodal EFNs have circular shape (Figures 3E-H). These showed distinct regions: the first region has cells with walls encrusted with suberin (Figures 2F, 3E-F) and is possible to observe the orifice connecting the secretory tissue and the outside environment where the nectar will be released (Figures 3E-F). In the second region, the secretory tissue is surrounded by phloem (Figure 3G) and a range of polygonal cells with suberin encrusted in its cell wall (Figures 2G, 3G).

Lastly, in a third region situated under the secretory tissue, a vascularization formed by xylem and phloem was observed (Figures 2H, 3H). Calcium oxalate druses were found surrounding all secretory tissue (Figure 3D).





**Figure 3.** Stipulates EFNs structure of *Caamembeca spectabilis*. A-C. Apical region. A. Epidermal cells with thick cutinized walls. B. Early development of central orifice. C. Formation by lysis of the canal that releases nectar. D-H. Polarized light. D. EFNs in the longitudinal section, note crystals. E. Apical region. F. Middle region. G. Basal region. H. Basal vascular of glands. (LT: Leaf trace, VT: Vascular trace, ST: Secretory tissue, PC: Polygonal cells, XL: Xylem, PL: Phloem). Scale bars: A, B, C: 50  $\mu\text{m}$ ; D: 80  $\mu\text{m}$ ; E-H: 50  $\mu\text{m}$ . "This figure is colored in the electronic version"

## DISCUSSION

The stipules are traditionally described as absent from Polygalaceae (Cronquist 1981). However, this study showed that the position and the vascularization of nodal glands originated in the leaf trace, in fact, are treated as modified stipules in *Caamembeca spectabilis*, as well as in *Caamembeca laureola* (cited as *Polygala laureola*) (Aguiar-Dias *et al.* 2011).

Modified stipules are found in many Angiosperm species allowing these structures to have other roles according the need and evolution of some groups (Weberling 2006). Our data corroborate Aguilar-Dias *et al.* (2011) suggesting that Polygalaceae may share this stipulate condition with legumes, despite not being a monophyletic character, i.e. in several genera and most species, nodal glands or any external trace of stipules were not detected.

The ontogeny of the EFNs have been mostly explained in terms of resource limitation for young plants to produce such traits (Miller 2007), or due to the architectural constraints associated with a plant's ability to attract a whole colony of ants (Boege 2005). However, to date, no study has assessed the anatomical and histological constraints promoting the observed ontogenetic patterns defences, particularly in EFNs (Villamil *et al.* 2013).

The recording of *Crematogaster* ants might indicate an interaction between plants and insects in *Caamembeca spectabilis*. These ants hunt both large and small prey such as grasshoppers, termites and wasps (Richard *et al.* 2001), which could play a role in combatting on plant-preying insects. The presence of a secretory structure that release exudate as reward to insects allows for the mutualistic relationship between plants and insects, especially ants (Vilhena-Potiguara *et al.* 2012).

The secretory tissue is delimited only by phloem, with the xylem staying below the nectariferous cells, features found by Aguilar-Dias *et al.* (2011). Francino *et al.* (2006) observed in *Chamaecrista trichopoda* (Caesalpinioideae) nectaries structurally similar as *C. spectabilis*, but also connected to xylem. Glands connected only at the phloem have larger carbohydrate concentrations in the nectar (Castro and Machado 2009).

The nectar is exuded by modified epidermal cells or trichomes, modified stomata or cuticle rupture (Fahn 1979). In *Caamembeca spectabilis* the nectar is released through a special pore formed by lysis. Probably in the secretory tissue, the sap drawn up from the phloem turns into pre-nectar and later in nectar, so it can be released out to the environment. Aguilar-Dias *et al.* (2011) did not mention suberin as a structure pertaining to EFNs of *Polygala laureola* (= *Caamembeca laureola*). In the present study, the large wall evidenced in the epidermal cells encrusted with suberin, in EFNs of *Caamembeca spectabilis*, is a new finding for this genus.

We observed fungi in the orifice which releases the exudate. We believe that the higher impregnation of suberin in epidermal cells allows us to infer that this layer prevents or hinders the spread of fungi to nectaries tissues. In particular, suberin has been known to reduce the loss of water from the aerial parts of the plant, restricting the apoplastic via of movement of water and solutes and forming a barrier to hinder the colonization of fungi (Rioux *et al.* 1995). The suberin layer also functions as secretion storeroom, protecting the adjacent cells against toxic compounds (Ascensão 2007).

Oxalate calcium druses were recorded in this study. These are among the most abundant plant in the vegetables' biomineral components (Weiner and Dove 2003). Druses is one type of calcium oxalate crystals and is particularly irritating to mouth and throat tissues when eaten (Prychid and Rudall 1999). Crystals are part of the structural protection of plants and also may be related to the elimination of excess calcium cytosolic, common in phloem in the proximity of the secretory tissues (Paiva and Machado 2005, Konyar *et al.* 2014).

## CONCLUSION

We have studied the ontogeny of the glands nodal in *Caamembeca spectabilis* and unveiled that these glands are linked to the leaves as stipular nectaries. In addition, we have presented new findings in the EFN structure and histology to help elucidate the mechanism of extrafloral nectar release in *Caamembeca spectabilis*, as well as give support to a better understanding of the morphology of nodal glands in *Caamembeca*.

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