



Distribution and structural aspects of extrafloral nectaries in leaves of *Pyrostegia venusta* (Bignoniaceae)

Mairon César Coimbra and Ana Hortência Fonsêca Castro *

Universidade Federal de São João del-Rei, Av. Sebastião Gonçalves Coelho, 400, 35501-296, Divinópolis, Minas Gerais, Brazil.
*Author for correspondence. E-mail: acaastro@ufsj.edu.br

ABSTRACT. *Pyrostegia venusta* (the orange trumpet or commonly called *cipó-de-São-João* in Brazil), a medicinal plant that grows with other plants, has an ecological importance due to the presence of nectaries on the leaves. The aim of this work was to study structural and histochemical aspects and the distribution of extrafloral nectaries (ENFs) in *P. venusta* leaves. Young leaves were collected, fixed and processed by usual techniques, and studied under light microscopy and scanning electron microscopy. Analyses showed that the extrafloral nectaries are dispersed throughout the leaf, with concentrations mainly in the basal third section. Nectaries lie in epidermal depressions and can be found in abaxial and adaxial leaf blades. Their morphology may be characterized macroscopically by achlorophyllous halos and microscopically as structures consisting of a short pedicel, oval multicellular head, covered by a thin cuticular layer with a secretory pole. Reducing sugars, neutral polysaccharides, proteins, starch and phenolic compounds were detected in extrafloral nectaries.

Keywords: ENFs, morphology, secretory structure.

Distribuição e aspectos estruturais de nectários extraflorais em folhas de *Pyrostegia venusta* (Bignoniaceae)

RESUMO. *Pyrostegia venusta* (cipó-de-São-João) é uma planta medicinal que cresce junto a outras plantas e que apresenta também importância ecológica, pela presença de nectários na região dos folíolos. O objetivo deste trabalho foi estudar aspectos estruturais, histoquímicos e da distribuição de nectários extraflorais (NEFs) em folhas de *P. venusta*. Folhas jovens foram coletadas, fixadas e processadas por técnicas usuais e estudadas sob microscopia de luz e eletrônica de varredura. As análises mostraram que os NEFs estão dispersos por toda a folha, concentrando-se preferencialmente no terço basal. Os nectários estão localizados em depressões epidérmicas e podem ser encontrados nas faces abaxial e adaxial da lâmina foliar. Macroscopicamente, os NEFs podem ser caracterizados como halos aclorofilados e microscopicamente, como estruturas com pedicelo curto, cabeça ovoide pluricelular, recobertos por uma fina camada cuticular e um polo secretor. Nos nectários extraflorais foram detectados açúcares redutores, polissacarídeos neutros, proteínas, amido e compostos fenólicos.

Palavras-chave: NEFs, morfologia, estrutura secretora.

Introduction

Nectaries may be classified, by their position, as floral (present in the floral parts) and extrafloral (present in the non-reproductive organs) (MARAZZI et al., 2013). According to Paiva (2011), the term “extrafloral” takes on a broader meaning and comprises any nectary that is not directly involved in the process of pollination, but rather in the protection of plants against herbivores (NASCIMENTO; DEL-CLARO, 2010).

Extrafloral nectaries (EFNs) are specialized glands or tissues that secrete nectar and are found on the vegetative portions of many plant species (DELGADO et al., 2011; MACHADO et al., 2008;

PAIVA; MACHADO, 2006). Secretion may be composed of sugars, amino acids, proteins and other compounds (HEIL, 2011). These nectar-secreting glands show external morphological diversity, distinct anatomical structures and different ways of releasing nectar. They occur on virtually all aboveground plant organs such as leaf blade, petiole, young stems, stipules, and on reproductive structures such as buds, calyx, inflorescence axis, flower peduncles and fruits. EFNs are well distributed among angiosperms and very common in the flora of the Brazilian savanna (MACHADO et al., 2008) where they seem to mediate interactions between insects and plants linked to defensive mutualisms (GONZALEZ, 2011). EFNs in

Bignoniaceae are conservative characters of a unified value, useful in generic delimitation (SEIBERT, 1948). Machado et al. (2008) describe morphologically the Bignoniaceae structures as flattened dishes that may be shifted upward or downward in relation to the level of leaf blade and peltate glandular trichomes.

Pyrostegia venusta (Ker Gawl.) Miers. (Bignoniaceae) (also known as *Pyrostegia ignea* and *Bignonia venusta*) is a liana creeper that easily adapts to hygrophyte and mesophyte environments. It is found at the edge of woods and fields, near the seaside and roadside, with a wide distribution throughout most of southern and southeastern Brazil. *P. venusta* leaves exhibit opposite phyllotaxy and are composed of two or three leaflets. In fact, it is an important component of the Brazilian savannah landscape (DUARTE; JURGENSEN, 2007; POOL, 2008). Even though considered a weed, it is not uncommon to see *P. venusta* as an ornamental plant, as it multiplies rapidly and may be used for adorning walls and pergolas. Its leaves and stems are employed in folk medicine as a tonic and to treat diarrhea, vitiligo, coughs and common infection-related diseases of the respiratory system (MOSTAFA et al., 2013).

The presence of EFNs in *Pyrostegia venusta* leaves was initially reported by Seibert (1948) as "glandular fields". The species has been recently listed in the World List of Angiosperms with extrafloral nectarines, updated by Keeler in 2010 (GONZALEZ, 2011). EFNs are structurally diverse and anatomically consist of a modified epidermis, with or without trichomes, and specialized parenchyma. Moreover, the tissue which constitutes the nectary is called nectariferous tissue (NEPI, 2007). According to Marazzi et al. (2013), in anatomically specialized EFNs, at least three different kinds of tissues may be recognized: the epidermis, the nectary parenchyma and the subnectary parenchyma. EFNs may be vascularized or non-vascularized structures. The most frequent vascularization consists of phloem or phloem and xylem (NEPI, 2007).

Currently, many studies are concerned with understanding the evolutionary significance of EFNs and their role in mutualist relationships involving insect-plant interactions neglecting their morphology, which is extremely important for taxonomic studies. Although much information on EFNS in different species of Bignoniaceae exists, the data related to the occurrence and morphology of these structures in *P. venusta* are limited and

practically non-existent. In current paper, the distribution and the structural and histochemical aspects of EFNs are studied to contribute towards the morphological characterization of these structures in *P. venusta* leaves.

Material and methods

Plant material

Young leaves of *Pyrostegia venusta* (Ker Gawl.) Miers (Bignoniaceae) located in the apical region of branches were collected in the Brazilian savanna or *cerrado*, in Divinópolis, Minas Gerais State, Brazil, at 20°10'45.9"S and 44°55'07.2"W. The voucher specimen was identified by Andréia Fonseca Silva of the Herbarium PAMG (PAMG 56307) of Agricultural Research Company of Minas Gerais.

Density of EFNs

Nectarines were observed immediately after collection with fresh and whole material. EFN density (EFNs cm⁻²) was estimated by fifteen fully expanded leaves (thirty leaflets) of each specimen under analysis (five individuals), with the determination of the limbus area and the number of nectarines, as proposed by Paiva and Machado (2006).

Leaves were analyzed under an Olympus SZ51 zoom stereo microscope to observe and count the nectarines, evaluate leaf area and determine nectary density. Data were analyzed by ANOVA tests and their means compared by Tukey's test at 5% significance. Results were given as mean ± standard deviation.

Light and scanning electron microscopy

For structural analysis, the leaves were fixed and stored directly in 70% alcohol (JOHANSEN, 1940). Hand-cut transverse and longitudinal sections of the fixed material were thus obtained. The series underwent the staining procedure with iodine green and Congo red. Sections were examined with a Zeiss Primo Star light microscope and documented by AxioVision.

Samples were SEM-observed (LEO Evo 40 XVP) by immersion in fixative (modified Karnovsky, 2.5% glutaraldehyde, 2.0% paraformaldehyde, 0.05 M cacodylate buffer, pH 7.2) for 24h, and prepared according to protocol by Bossola and Russell (1999).

Histochemical analyses of EFNs

The following histochemical methods were used on the fresh material: ferric chloride for phenolic compounds; zinc chloride iodine for lignin and

starch (JENSEN, 1962); Sudan III (SASS, 1951) and IV (GERLACH, 1984) for lipids in general; Lugol for the identification of starch (JOHANSEN, 1940); bromophenol blue for proteins (MAZIA et al., 1953); Schiff reagent (PAS) for neutral polysaccharides; Fehling's reagent for reducing sugars (SASS, 1951).

Results and discussion

ENFs of *P. venusta* were characterized macroscopically by achlorophyllous halos with approximately 0.80 ± 0.11 mm in diameter, randomly distributed throughout the leaf blade (Figures 1a and b). Density was 5.27 ± 1.74 ENFs cm^{-2} and the occurrence of these structures in the basal third section of the leaflet was 6.39 ± 2.10 , while this measurement in the middle and apical third sections was 4.30 ± 1.93 and 3.53 ± 1.11 , respectively (Figure 1c). ENFs were microscopically identified in the adaxial and abaxial surfaces as small epidermal depressions (Figure 1d). *P. venusta*'s ENFs could be morphologically characterized as peltate glandular trichomes with short pedicel and multicellular ovoid heads, covered by a thin layer of cuticle and provided with a secretory pole (Figure 1d, e and f). The information obtained by scanning electron microscopy corroborated the report made under light microscopy (Figures 1g and h).

The presence of ENFs on leaves in the species studied may be the result of evolutionary adaptation and may be explained by the fact that *P. venusta* is a liana vine, which facilitates access of herbivores and other predators to the plant bodies, because it requires brackets (walls and other plants) for their growth that allow access to ENFs. As suggested by Chamberlain and Holland (2009), staining some ENFs may provide a visual aid for revealing the location of these structures to some animals, which cannot be eliminated, such as in the case of ENFs in *P. venusta*.

The randomization, the insertion of ENFs in epidermal depressions and their presence in the adaxial and abaxial surfaces may significantly reduce exudate loss by evaporation, as suggested by Paiva and Machado (2006). The above increases their availability of secretions and facilitates the patrol of limbus leaflets by insects, which contributes to the reduction of herbivore pests (HEIL; KOST, 2006; KOST; HEIL, 2005; PAIVA; MACHADO, 2006). The random distribution of ENFs and their preferential accumulation at the basal regions of the leaflet have been reported in *Hymenaea stigonocarpa* Mart. ex-Hayne (Fabaceae) (PAIVA; MACHADO, 2006).

Morphologically, the nectaries found in *P. venusta* corroborate the description by Seibert (1948) and Machado et al. (2008) for the Bignoniaceae family. Gonzalez (2011) recently reported the presence of these structures in different species of the family Bignoniaceae, including the species studied in current research. Duarte and Jurgensen (2007) refer to ENFs of *P. venusta* only as glandular trichomes, which are not related to the interactions between insects and plants. However, glandular trichomes and ENFs are distinct secretory structures (EVERT, 2013). The glandular trichomes are epidermal appendix originating in protoderm cells, whereas cells subjacent to tissues such as parenchyma or even vascular tissues also participate in the ontogeny of ENFs (ASCENSÃO, 2007; EVERT, 2013). According Ascensão (2007), it is highly difficult to distinguish between these two types of structures, often referred to generically as glandular trichomes, without ontogenetic studies. Other structures involved in plant interactions, such as colleters, food bodies and similar structures are also considered trichomes in some species (PAIVA; MACHADO, 2006). Denardi et al. (2012) reports that the glandular trichomes in *Connarus suberosus* function as food bodies because of their presence on the exposed surfaces of developing organs and the chemical nature of their reserve products.

ENFs in *P. venusta* are similar to flattened dishes with a secretory pole and a more elevated cupule sunk on the leaf surface (Figure 1d, g and h). These nectaries are named scale-like nectaries (MACHADO et al., 2008). The secretory pole has an upper secretory region, formed by one layer of cells covered by a thin cuticle and by an underlying region with one layer of isodiametric epidermal cells connected to the leaf parenchyma (Figure 1d). The nectary parenchyma's cells are not anatomically distinguished from other cells of the parenchyma and no vascularization was reported in these structures.

In the histochemical characterization of ENFs, a positive reaction of ferric chloride (Figure 2b), zinc chloride iodine (Figure 2c), bromophenol blue (Figure 2d), Periodic Acid Schiff (Figure 2e) and Fehling (Figure 2f) revealed the presence of phenolic compounds, starch, proteins, neutral polysaccharides and reducing sugars, respectively. Tests conducted with lugol, Sudan III and Sudan IV did not indicate the presence of starch and lipids.

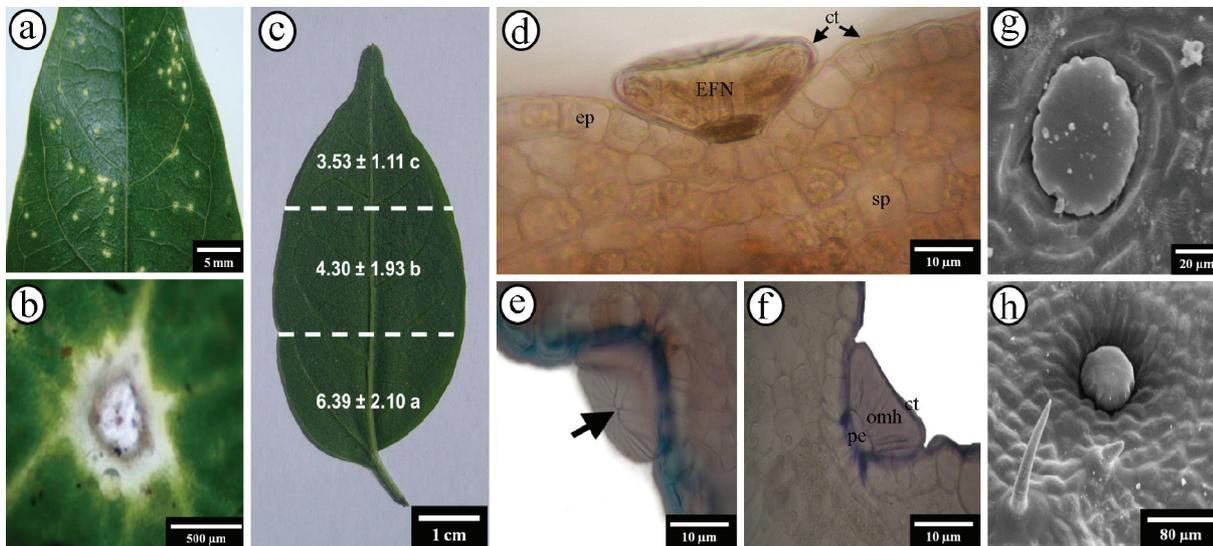


Figure 1. EFNs in *P. venusta*. a) Adaxial side of leaflets showing random distribution of EFNs; b) Details of EFN in the abaxial surface; c) Distribution of EFNs on the leaflets; d) Cross-section of leaflet showing EFN, epidermis (ep), sponge parenchyma (sp) and cuticle (ct); e) EFN with secretory pole (black arrow); f) Overview of EFN in cross-section; Note the pedicel (pe), oval multicellular head (omh) and cuticle (ct); g and h) Image scanning electron microscopy; Note the location of EFN in the epidermal depression in h.

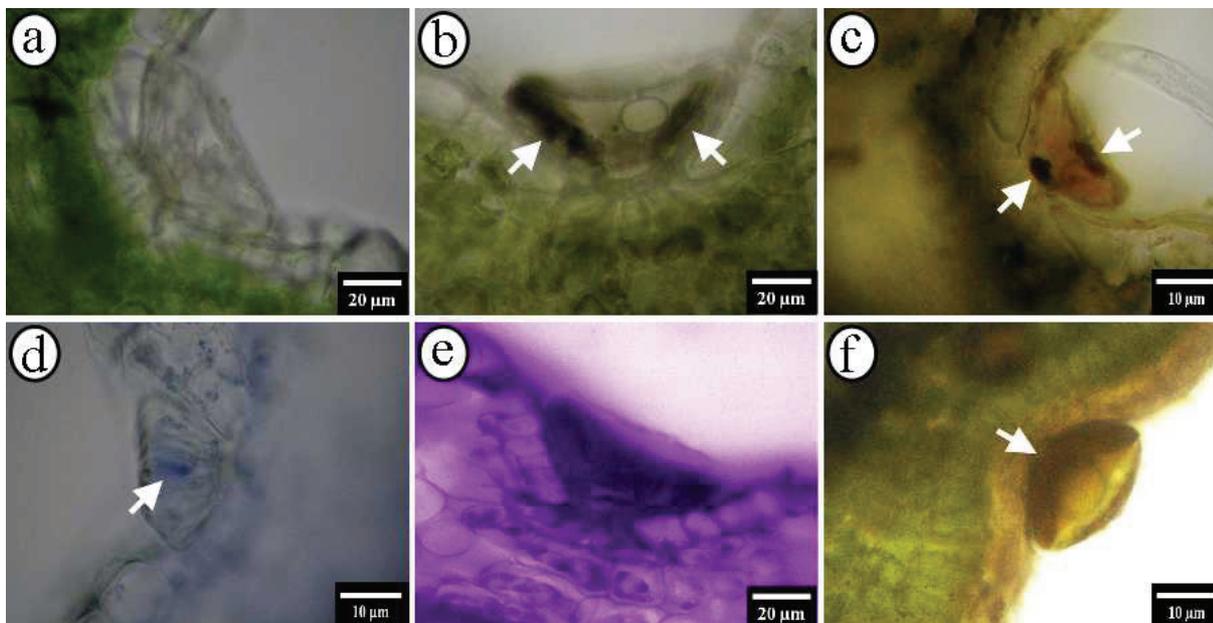


Figure 2. Histochemistry of EFNs *P. venusta*. Cross-section of leaflet showing the nectary (arrow indicates sites of positive reaction). a) Control; b) Ferric Chloride test; c) Zinc Chloride Iodine test; d) Bromophenol Blue test; e) Periodic Acid Schiff test; f) Fehling test.

According to Pimentel et al. (2011), the detection of different chemicals in the cells of nectaries does not imply their presence in the secretion, although nectaries still have ecologically potential functions. The presence and availability of different metabolite groups in EFNs may serve as attractive and nutritional supplements for different insects, such as hymenopterans (ants and others) and homopterans, a well-discussed fact in the literature (NOGUEIRA et al., 2012). The nectaries attract ants that protect the leaves against herbivores (CHAMBERLAIN;

HOLLAND, 2009). In return for extrafloral nectar, ants are the bodyguards and patrol young leaf surfaces, removing or deterring unwanted pests (BIXENMANN et al., 2011; ROSUMEK et al., 2009). Phenolic compounds identified in nectaries cells of the species under analysis may provide protection against herbivores, microorganisms, and excess ultraviolet radiation. They may also protect the protoplast maintaining cellular integrity when subjected to water stress (PAIVA; MACHADO, 2008). Although the presence of plastids with starch

grains in nectariferous tissues have been extensively reported in the literature (ROCHA; MACHADO, 2009), these compounds in the EFNs of *P. venusta* were detected only in the reaction with zinc chloride iodine. Starch accumulated in the plastids may be the source of some components of nectar which is total or partially degraded during manifestations of secretory activity (HEIL, 2011). It is likely that sugars detected in *P. venusta*'s nectaries originate from degradation starch grain present in plastids, as has been reported by Pimentel et al. (2011) on the secretion structures of *Pavonia alnifolia* (Malvaceae). Detected proteins and polysaccharides suggest that such compounds might be found in exudates, which is an evidence of the nectar's complexity (COUTINHO et al., 2012). These substances in EFNs have also been described by other authors (COUTINHO et al., 2010; ROCHA et al., 2009).

Conclusion

The present study is an initial investigation on the distribution and morphology of EFNs in *P. venusta*. Since the nectaries presented close vascularization, further anatomical studies must be developed to carefully examine and describe the relationships between these nectaries and the leaves vascular systems.

Acknowledgements

The authors would like to thank Professor Eduardo Alves for providing the Laboratory of Electron Microscopy and Ultra Structural Analysis, Department of Plant Pathology/Federal University of Lavras, to perform the analyses under scanning electron microscopy.

References

- ASCENSÃO, L. Estruturas secretoras em plantas: uma abordagem morfoanatômica. In: FIGUEIREDO, A. C.; BARROSO, J. G.; PEDRO, L. G. (Ed.). **Potencialidades e aplicações das plantas aromáticas e medicinais**. Lisboa: Faculdade de Ciências de Lisboa, 2007. p. 19-28.
- BIXENMANN, R. J.; COLEY, P. D.; KURSAR, T. A. Is extrafloral nectar production induced by herbivores or ants in a tropical facultative ant-plant mutualism? **Oecologia**, v. 165, n. 2, p. 417-425, 2011.
- BOSSOLA, J. J.; RUSSELL, L. D. **Electron Microscopy**. Boston: Jones and Bartlett Publishers, 1999.
- CHAMBERLAIN, S. A.; HOLLAND, J. N. Quantitative synthesis of context dependency in ant – plant protection mutualisms. **Ecology**, v. 90, n. 8, p. 2384-2392, 2009.
- COUTINHO, Í. A. C.; VALENTE, V. M. M.; MEIRA, R. M. S. A. Ontogenetic, anatomical and histochemical study of the extrafloral nectaries of *Sapium biglandulosum* (Euphorbiaceae). **Australian Journal of Botany**, v. 58, n. 3, p. 224-232, 2010.
- COUTINHO, Í. A. C.; FRANCINO, D. M. T.; AZEVEDO, A. A.; MEIRA, R. M. S. A. Anatomy of the extrafloral nectaries in species of *Chamaecrista* section *Absus* subsection *Baseophyllum* (Leguminosae, Caesalpinioideae). **Flora**, v. 207, n. 6, p. 427-435, 2012.
- DELGADO, M. N.; SILVA, L. C.; BÁO, S. N.; MORAIS, H. C.; AZEVEDO, A. A. Distribution, structural and ecological aspects of the unusual leaf nectaries of *Calolisianthus species* (Gentianaceae). **Flora**, v. 206, n. 7, p. 676-683, 2011.
- DENARDI, J. D.; OLIVEIRA, D. M. T.; PAIVA, E. A. S. Glandular trichomes in *Connarus suberosus* (Connaraceae): distribution, structural organization and probable functions. **Revista de Biologia Tropical**, v. 60, n. 1, p. 505-513, 2012.
- DUARTE, M. R.; JURGENSEN, I. Diagnose morfoanatômica de caule e folha *Pyrostegia venusta* (Ker Gawl.) Miers, Bignoniaceae. **Latin American Journal of Pharmacy**, v. 26, n. 1, p. 70-75, 2007.
- EVERT, R. F. **Anatomia das plantas de Esau**. São Paulo: Editora Blucher, 2013.
- GERLACH, D. **Botanische mikrotechnik**. Stuttgart: Georg. Thieme Verlag, 1984.
- GONZALEZ, A. M. Domacios y nectarios extraflorales en Bignoniáceas: componentes vegetales de una interacción mutualística. **Boletín de la Sociedad Argentina de Botánica**, v. 46, n. 3-4, p. 271-288, 2011.
- HEIL, M. Nectar: generation, regulation and ecological functions. **Trends in Plant Science**, v. 16, n. 4, p. 191-200, 2011.
- HEIL, M.; KOST, C. Priming of indirect defences. **Ecology Letters**, v. 9, n. 7, p. 813-817, 2006.
- JENSEN, W. A. **Botanical Histochemistry: principles and practice**. San Francisco: W. H. Freeman, 1962.
- JOHANSEN, B. A. **Plant microtechnique**. New York: Mc Graw-Hill, 1940.
- KOST, C.; HEIL, M. Increased availability of extrafloral nectar reduces herbivory in Lima bean plants (*Phaseolus lunatus*, Fabaceae). **Basic and Applied Ecology**, v. 6, n. 3, p. 237-248, 2005.
- MACHADO, S. R.; MORELLATO, L. P. C.; SAJO, M. G.; OLIVEIRA, P. S. Morphological patterns of extrafloral nectaries in woody plant species of the Brazilian cerrado. **Plant Biology**, v. 10, n. 5, p. 660-673, 2008.
- MARAZZI, B.; BONSTEIN, J. L.; KOPTUR, S. The diversity, ecology and evolution of extrafloral nectaries: current perspectives and future challenges. **Annals of Botany**, v. 111, n. 6, p. 1243-1250, 2013.
- MAZIA, D.; BREWER, P. A.; ALFERT, M. The cytochemistry staining and measurement of protein with mercuric bromophenol blue. **Biological Bulletin**, v. 104, n. 1, p. 57-67, 1953.
- MOSTAFA, N. M.; EL-DAHSHAN, O.; SINGAB, A. N. B. *Pyrostegia venusta* (Ker Gawl.) Miers: a botanical, pharmacological and phytochemical review. **Medicinal and Aromatic Plants**, v. 2, n. 3, p. 1-6, 2013.

- NASCIMENTO, E. A.; DEL-CLARO, K. Ant visitation to extrafloral nectaries decreases herbivory and increases fruit set in *Chamaecrista debilis* (Fabaceae) in a Neotropical savanna. **Flora**, v. 205, n. 11, p. 754-756, 2010.
- NEPI, M. Nectary structure and ultrastructure. In: NICOLSON, S. W.; NEPI, M.; PACINI, E. (Ed.). **Nectaries and nectar**. Dordrecht: Springer, 2007. p. 129-166.
- NOGUEIRA, A.; REY, P. J.; LOHMANN, L. G. Evolution of extrafloral nectaries: adaptive process and selective regime changes from forest to savanna. **Journal of Evolutionary Biology**, v. 25, n. 11, p. 2325-2340, 2012.
- PAIVA, E. A. S. Petaline nectaries in *Swietenia macrophylla* (Meliaceae): Distribution and structural aspects. **Flora**, v. 206, n. 5, p. 484-490, 2011.
- PAIVA, E. A. S.; MACHADO, S. R. Ontogênese, anatomia e ultra-estrutura dos nectários extraflorais de *Hymenaea stigonocarpa* Mart. ex Hayne (Fabaceae – Caesalpinioideae). **Acta Botanica Brasilica**, v. 20, n. 2, p. 471-482, 2006.
- PAIVA, E. A. S.; MACHADO, S. R. The floral nectary of *Hymenaea stigonocarpa* (Fabaceae, Caesalpinioideae): structural aspects during floral development. **Annals of Botany**, v. 101, n. 1, p. 125-133, 2008.
- PIMENTEL, R. R.; MACHADO, S. R.; ROCHA, J. F. Estruturas secretoras de *Pavonia alnifolia* (Malvaceae), uma espécie ameaçada de extinção. **Rodriguésia**, v. 62, n. 2, p. 253-262, 2011.
- POOL, A. A review of the genus *Pyrostegia* (Bignoniaceae). **Annals of the Missouri Botanical Garden**, v. 95, n. 3, p. 495-510, 2008.
- ROCHA, J. F.; MACHADO, S. R. Anatomy, ultrastructure and secretion of *Hibiscus pernambucensis* Arruda (Malvaceae) extrafloral nectary. **Revista Brasileira de Botânica**, v. 32, n. 3, p. 489-498, 2009.
- ROCHA, D. I.; SILVA, L. C.; VALENTE, V. M. M.; FRANCINO, D. M. T.; MEIRA, R. M. S. A. Morphoanatomy and development of leaf secretory structures in *Passiflora amethystina* Mikn (Passifloraceae). **Australian Journal of Botany**, v. 57, n. 7, p. 619-626, 2009.
- ROSUMEK, F. B.; SILVEIRA, F. A. O.; NEVES, F.; BARBOSA, N. P.; DINIZ, L.; OKI, Y.; PEZZINI, F.; FERNANDES, G. W.; CORNELISSEN, T. Ants on plants: a meta-analysis of the role of ants as plant biotic defenses. **Oecologia**, v. 160, n. 3, p. 537-549, 2009.
- SASS, J. E. **Botanical Microtechnique**. Ames: Iowa State College Press, 1951.
- SEIBERT, R. J. The use of gland in a taxonomic consideration of the family Bignoniaceae. **Annals of the Missouri Botanical Garden**, v. 35, n. 2, p. 123-136, 1948.

Received on April 2, 2014.

Accepted on May 13, 2014.

License information: This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.