

WHAT ARE THE “STICKY RINGS” ON STEMS OF *ANULOCAULIS* AND RELATED TAXA (NYCTAGINACEAE) FROM ARID REGIONS?

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ABSTRACT

Anulocaulis, commonly known as “ringstem,” is a small, unusual genus restricted to the Chihuahuan, Sonoran, and Mojave deserts of North America. Here we combined light microscopy and histochemical tests to characterize for the first time the “sticky structures” (here called secretory rings) found on the stem internodes of *Anulocaulis*. The secretory rings were shown to be groups of epidermal cells, or unicellular glandular trichomes, which largely differ from their neighboring cells both in structure and histochemistry. The cells start to differentiate in early stages of stem development. They begin as regular epidermal cells, but later their anticlinal and external tangential walls start to enlarge. At maturity the cells become remarkably elongated, even balloon-like, with dense cytoplasmic content. Although the secretory rings have been reported as “mucilaginous structures” based on morphological observations, preliminary histochemical analyses showed that its exudate is complex, including a mixture of mucilage, proteins, and phenolic compounds. Future investigations are needed to compare the anatomy of the secretory rings within related genera of Nyctagineae and characterize the chemical components of their exudate more specifically to search for potential homologies and adaptive functions of these structures.

RESUMEN

Anulocaulis, comúnmente conocido como “ringstem,” es un género pequeño que se encuentra restringido a los desiertos de Chihuahua, Sonora y Mojave de América del Norte. En este estudio, usamos microscopía óptica y pruebas histoquímicas para caracterizar por primera vez las “sticky structures” (aquí denominadas “secretory rings”) que se encuentran en los entrenudos del tallo de *Anulocaulis*. Se demostró que los anillos secretores son grupos de células epidérmicas, o tricomas glandulares unicelulares, que se diferencian en gran medida de sus células vecinas tanto en estructura como en histoquímica. Las células comienzan a diferenciarse en las primeras etapas del desarrollo del tallo. Comienzan como células epidérmicas normales, pero luego sus paredes anticlinal y tangencial externa comienzan a agrandarse. En la madurez, las células se vuelven notablemente alargadas, incluso como globos, con un contenido citoplasmático denso. Aunque los anillos secretores han sido descritos como “estructuras mucilaginosas” basadas en observaciones morfológicas, los análisis histoquímicos preliminares mostraron que su exudado es complejo, incluyendo una mezcla de mucilago, proteínas y compuestos fenólicos. Son necesarias investigaciones futuras que permitan estudiar comparativamente la anatomía de los anillos secretores en los géneros de Nyctagineae y así mismo caracterizar los componentes químicos de su exudado más específicamente para buscar posibles homologías y funciones adaptativas de estas estructuras.

KEY WORDS: Anatomy, Caryophyllales, Nyctagineae, secretory structures, Chihuahuan Desert, glandular trichomes

INTRODUCTION

Anulocaulis is a small genus of perennial herbs with just five species. It is included in tribe Nyctagineae, which is the largest and most diverse tribe in the family Nyctagineae (Douglas & Spellenberg 2010). The genus is endemic to arid regions of North America (e.g., Chihuahuan, Sonoran, and Mojave Deserts) and is distributed from northern Mexico to southeastern California in the United States of America (Spellenberg 1993; Douglas & Spellenberg 2010). *Anulocaulis* may be divided into two groups based on fruit morphology (Spellenberg 1993). The first encompasses *A. annulatus*, *A. hintoniorum*, and *A. eriosolenus*, which have smooth anthocarps. *Anulocaulis annulatus* is restricted to low, hot elevations near Death Valley in the Mojave Desert, while *A. hintoniorum* and *A. eriosolenus* are restricted to the Chihuahuan Desert in Mexico and Texas. The second group comprises *A. leiosolenus* (considered to have four varieties, Spellenberg 1993) and *A. reflexus*. These are characterized by their variously winged and wrinkled anthocarps and are primarily gypsum endemic plants

(Spellenberg 1993). Taxa in this group are mainly Chihuahuan, with three disjunct population centers in Arizona and Nevada (Moore et al. 2014).

Anulocaulis was segregated from the large and diverse genus *Boerhavia* by Standley in 1909 based on several morphological characteristics, including the presence of the “sticky rings.” Since the recognition of the genus *Anulocaulis* by Standley (1909), several authors have noted the presence of this structure (Fig. 1) encircling a small portion of the internodes (Bittrich & Kühn 1993; Spellenberg 2003; Hernández-Ledesma et al. 2010). In fact, the name *Anulocaulis* was given by Standley in reference to the presence of such structures (*anulo* = ring; *caulis* = stem), which he described as “stems glabrous, but the middle of each internode usually provided with a reddish ring which exudes a mucilaginous fluid.” For this reason, *Anulocaulis* species are popularly known as “ringstem.” Various terms have been used to address this structure, such as “sticky bands” (Spellenberg 1993, 2003), “glandular rings” (Bittrich & Kühn 1993), “sticky rings” (McClellan & Boecklen 1993), “internodal bands of viscid secretions” or “viscid bands” (Douglas & Manos 2007), and “mucilaginous rings” or “glutinous bands” (Hernández-Ledesma et al. 2010); here we will use the term “secretory rings.” These secretory rings are observed not only in *Anulocaulis* but also in several species of *Boerhavia* (e.g., *B. erecta*, *B. spicata*, & *B. xantii*) and the monotypic genus *Cyphomeris* (Bittrich & Kühn 1993; Douglas & Manos 2007; Hernández-Ledesma et al. 2010).

The occurrence of sticky structures/secretion onto plant surfaces received attention in the last few years due to several lines of evidence indicating that they can mediate important aspects of the ecology of species with such secretions (Krimel & Pearse 2013; LoPresti 2015; Karban et al. 2019). Sticky exudates on plant surfaces affect other organisms, including herbivores or their predators (LoPresti 2015). In many species, the sticky substances derive from glandular trichomes, which are typically considered direct defenses against herbivores (Krimel & Pearse 2013). In some groups, the sticky structures/secretion function as traps that catch insects, which become available to predatory insects that defend the plants against herbivores. Effectively, this represents an indirect defense system that ultimately increases plant fitness (Karbon et al. 2019). For example, in *Nicotiana attenuata*, where the flowers’ components are relatively sticky, the number of insect carrion found on plants positively predicted both the number of predators attracted, whereas the number of predators positively correlated with the number of seed capsules produced by the plant (Karbon et al. 2019). Besides *N. attenuata* (Solanaceae), similar cases of sticky plants have been reported in different groups, including Asteraceae (*Maedia elegans*, *Hemizonia congesta*—Krimel & Pearse 2013; LoPresti et al. 2018) and Ranunculaceae (*Aquilegia eximia*—LoPresti et al. 2015). Such interactions between sticky plants and predators can be compared with better-known mutualistic cases such as plants that provide predators with nutritious resources (e.g., nectar, pollen, protein bodies; Krimel & Pearse 2013; Karban et al. 2019). In Nyctaginaceae, as in other groups, the occurrence of sticky substances has also been related to plant defense in another way, which is sand entrapment on the plant surface (LoPresti & Karban 2016). Plants showing this mechanism, termed psammophory, were reported as suffering less damage from chewing herbivores, as demonstrated in *Abronia latifolia* (Nyctaginaceae) and other species (LoPresti & Karban 2016), and herbivore performance was also reduced (LoPresti et al. 2017). Most relevant to the present case, rings in *Boerhavia* “*spicata*” (also Nyctaginaceae; in actuality the plants were *B. torreyana*, R. Spellenberg, per. comm.) were shown to reduce aphid infestation (McClellan & Boecklen 1993).

Despite the peculiarity of the secretory rings in Nyctaginaceae species, a structural investigation of this character is still lacking. Therefore, this work aimed to analyze the anatomy and histochemistry of the secretory rings in stems of *Anulocaulis leiosolenus* in order to characterize the secretory structure and determine the main chemical compounds present in the exudate. By doing this, we expect to shed light on what these secretory rings truly are so that in further investigations it would be possible to search for potential homologies and the adaptive function of these structures.

MATERIALS AND METHODS

Stem samples were obtained at four sites in the northern Chihuahuan Desert, in the states of Texas and New

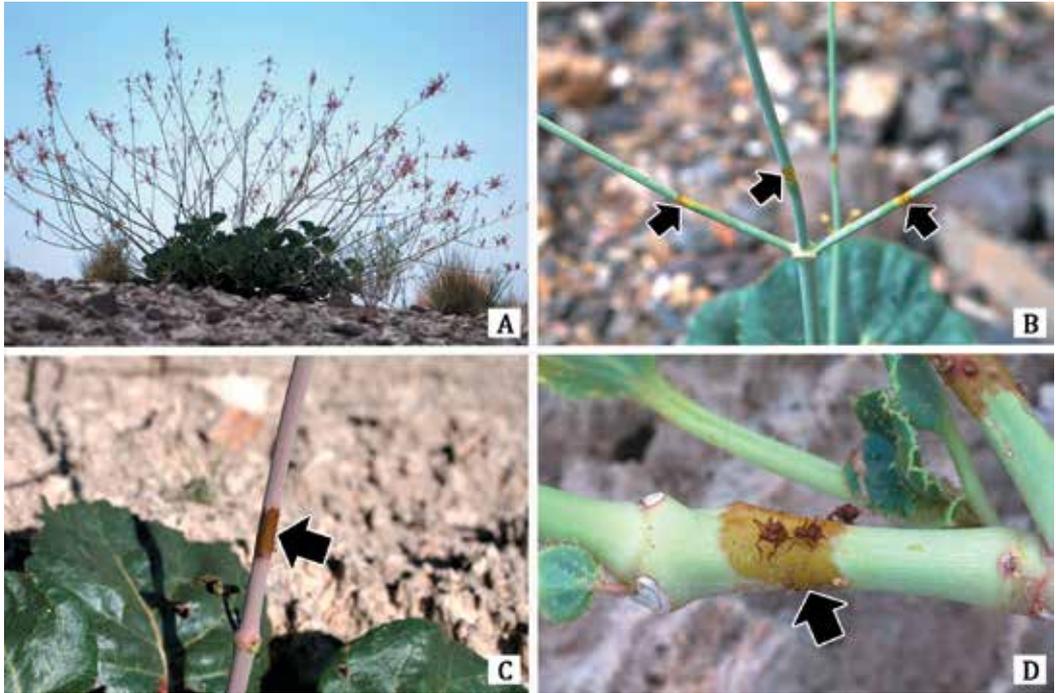


FIG. 1. Morphological aspects of the plant and secretory rings in *Anulocaulis leiosolenus* var. *gypsogenus*. **1a:** Habit. **1b:** Secretory rings in young internodes (arrows). **1c:** Detail of secretory ring in older internode. Notice the irregular contour of the rings. **1d:** Secretory ring with immobilized insects (arrow).

Mexico, United States of America, from 12–17 Sep 2018. In total, we collected five specimens of *Anulocaulis leiosolenus* var. *gypsogenus*, three of *Anulocaulis leiosolenus* var. *leiosolenus* and two of *Cyphomeris gypsophiloides* (see Appendix 1 for detailed information on the collection, collectors, localities, and herbarium vouchers).

The secretory rings are variable in their expression either at species or population levels. Although all the species and varieties collected have been reported with these structures, only the representatives of *Anulocaulis leiosolenus* var. *gypsogenus* from the population at Yeso Hills (Eddy County, New Mexico, USA) showed them at the time we visited the population. Therefore, specimens from this population were used for the anatomical and histochemical study.

Stems of all individuals were fixed in 70% isopropanol and later stored in 70% ethanol. For the anatomical study, freehand sections were initially performed to identify the regions with the cells forming the secretory rings. Later, the material was dehydrated in butanol series (Johansen 1940) and embedded in Paraplast or dehydrated in an ethanol series and embedded in 2-hydroxyethyl-methacrylate (historesin - Histosec, Merck). The samples were sectioned in transversal and longitudinal planes in a rotary microtome at ca. 5–10 μm thickness. Sections performed by hand or embedded in Paraplast were stained with Safrablau (Bukatsch 1972), whereas sections embedded in historesin were stained in Toluidine Blue (O'Brien et al. 1964). Permanent slides were produced using Permount (Fisher Scientific, Pittsburgh, USA), and photomicrographs were taken using a light microscope (Leica DMLB).

Freehand sections were obtained from fixed material, and the following tests were performed following Demarco (2017): Sudan Black and Sudan IV staining for detection of lipids; Nile blue for acid/neutral lipids; Ruthenium red, Alcian blue, and tannic acid and ferric chloride for acid mucilage and/or pectins; Lugol reagent for starch; PAS reaction (periodic acid: Schiff's reagent) for carbohydrates; Aniline Blue Black staining for proteins; NADI reaction for terpenes; ferric chloride and potassium dicromate staining for phenolic compounds;

phloroglucinol staining for lignin; and Wagner's reagent for alkaloids. Standard control procedures were carried out simultaneously as required for each test.

RESULTS

In *Anulocaulis leiosolenus* var. *gypsogenus*, the secretory rings occur in young and mature stem internodes (Fig. 1). Morphologically, they appear as irregular marks that are yellowish-green to brown due to secreted material (Fig. 1B–D). In the field, the secretion can appear when the internode is young, and expands as the stem matures, and is evidently self-limiting, as older rings sometimes become covered in sand, insects, or other debris (Fig. 1D). The secretion is apparently viscid at all times; furthermore, we have observed that herbarium specimens that are several decades old remain quite sticky.

In early stages of stem development, approximately at the second or third internode, the secretory rings have already started to differentiate (Fig. 2A–C). These structures consist of a group of differentiated epidermal cells, which can be characterized as unicellular glandular trichomes (Fig. 2A–C). Initially, they are cells with regular size and shape that later begin to elongate their anticlinal and/or distal tangential walls (Fig. 2C). As this structure continues to develop with the increase in diameter of the stem, cells with much larger size are gradually found side by side (Fig. 2C, 2F), forming either a complete ring (Fig. 2D) or an incomplete ring as seen in external morphology (Fig. 1C–D). In developed secretory rings, the epidermal cells are markedly enlarged and radially elongated into palisade-like cells, sometimes assuming an ovoid or balloon-like shape, overlying much smaller and isodiametric subepidermal cells (Fig. 2D–E).

The developed cells forming the secretory rings exhibit a large nucleus (Fig. 2I–J), a dense cytoplasm (Fig. 2D–J), and a characteristic thickened primary wall, especially in the distal tangential walls (Fig. 2I–J). In unstained sections, the content shows a brownish color (Fig. 3A). This content turns dark red when stained with safranin and Astra Blue (Fig. 2E), light to dark green when stained by Safrablau in free-hand sections (Fig. 2G–H), and light blue when stained with Toluidine Blue (Fig. 2I–J). The content secreted by these cells is the protoplasm itself (Fig. 2E, 2G–J).

The thickened primary cell walls of secretory ring cells were found to be rich in pectins, as indicated by the light pinkish color in sections stained with Toluidine Blue (Fig. 2J, 3B). The secretion reacted positively (brown color) to ferric chloride (Fig. 3C), which typically indicates the presence of general, non-structural phenolic compounds. The presence of mucilages was revealed by a positive reaction (light blue color) with Alcian blue (Fig. 3D), whereas Aniline Blue Black staining detected the presence of nonstructural proteins (dark color) (Fig. 3E). Therefore, the exudate of these cells showed a mixture of mucilage, proteins, and phenolic compounds.

DISCUSSION

In this study, the ontogeny, structure, and histochemistry of the secretory rings found in *Anulocaulis* are described for the first time. We found that the rings are, in fact, a group of epidermal cells that differentiate early in development and which produce, store, and release a complex secretion, consisting of mucilage, phenolic compounds, and proteins. The cells forming the secretory rings are markedly different from the surrounding cells (e.g., regular epidermal cells and subepidermal cells) both in structure and in metabolism. Structurally, the secretory rings are formed by a layer of differentiated epidermal cells, which are notably larger and elongated outwards. These cells resemble unicellular trichomes or papillose cells, which are highly variable epidermal appendages, whose distinction is not always clear (Evert 2006; Rudall 2007). Another feature that distinguishes these cells is their remarkably dense cytoplasm, which might be rich in organelles, as commonly observed in cells with active physiological roles in the secretion of secondary metabolites (Fahn 1979; Evert 2006; Tilney et al. 2014; Fernandes et al. 2017; Ballego-Campos & Paiva 2018). Also, the histochemical detection of proteins in the cytoplasm of the secretory rings may also indicate high metabolic activity within these cells (Fernandes et al. 2017).

The identification of mucilage in the exudate of the secretory rings confirms field observations predicting

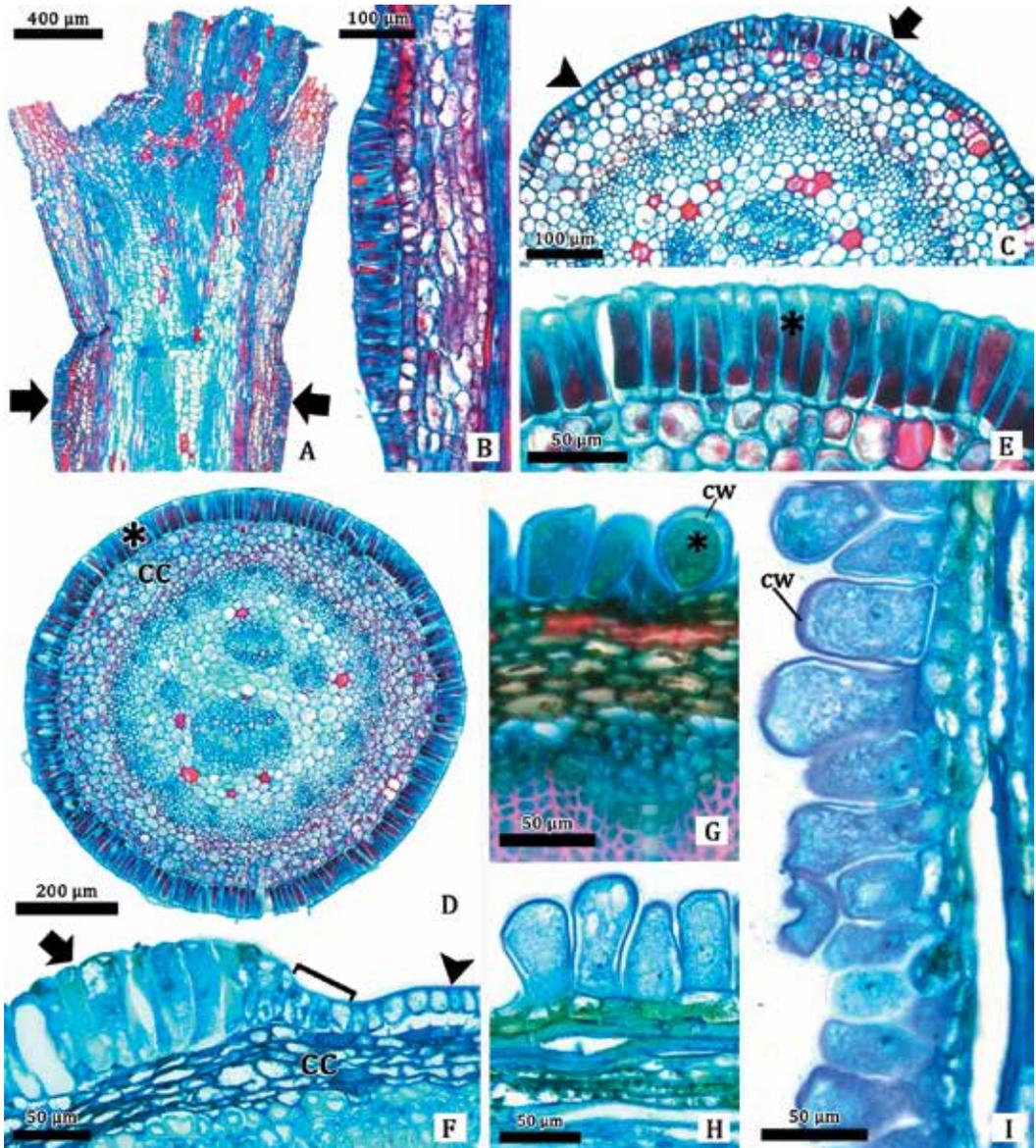


Fig. 2. Development and anatomy of secretory rings in *Anulocaulis leiosolenus* var. *gypsogenus*. A–B, I: Longitudinal sections. C–H: Transverse sections. **2a–b**: Early developmental stage of stem internodes showing the beginning on the differentiation of secretory epidermal cells (arrows). **2c**: Stem section showing that cells become gradually enlarged and radially elongated (arrow), while other cells remain still regular (arrowhead). **2d–e**: Secretory ring occupying the entire stem internode circumference. Notice the enlarged and elongated epidermal cells with dense and homogenous content (asterisk) underlying smaller and isodiametric cortical cells (cc). **2f**: Detail of secretory ring showing transition region between regular (arrowhead), enlarging (bracket), and elongated epidermal cells (arrow). **2g–h**: Free-hand sections stained with Saffrablau showing epidermal cells forming the secretory rings with dense and homogeneous content (asterisk). Notice the thickened cell wall (cw), especially in the external periclinal walls (g) and disrupted cells (thin arrow) (h), a possible mechanism for secretion releasing. **2i**: Developed epidermal cells showing dense cytoplasm, large nucleus, and thickened walls. **2j**: Detail of secretory ring in longitudinal view showing also the balloon-like shape of some cells which have also dense cytoplasm, large nucleus, and thickened walls (cw). Notice their thickened wall in pinkish color by the Toluidine Blue staining, evidence of the presence of pectins.

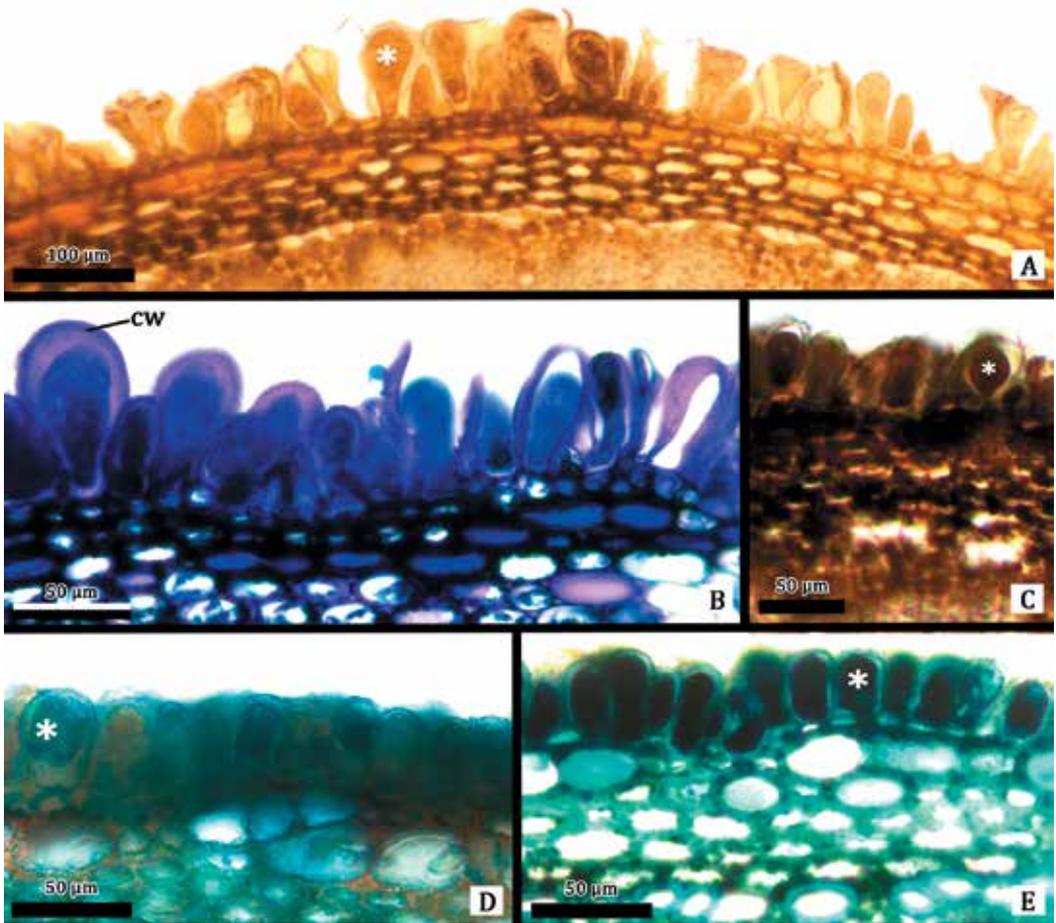


FIG. 3. Histochemical characterization of secretory rings in *Anulocaulis leiosolenus* var. *gypsogenus*. **3a**: Unstained section showing cells forming the secretory ring with balloon-like shape and yellowish to brownish content (asterisk). **3b**: Sample treated with Toluidine Blue. Thickened cell walls (cw) stain pink, thus indicating the presence of pectins. **3c**: Positive result for ferric chloride evidencing the presence of phenolic compounds (asterisk). **3d**: Mucilage (asterisk) detected by with Alcian Blue reagent. **3e**: Positive reaction for protein (asterisk) using Aniline Blue Black.

that these structures would contain mucilaginous components (Bittrich & Kühn 1993; Spellenberg 1993, 2003; Hernández-Ledesma et al. 2010). However, given that the secretory rings produce a complex exudate they should not be classified as “mucilaginous rings” as mentioned by Hernández-Ledesma et al. (2010). Typical mucilaginous cells, as the ones reported to occur in reproductive (e.g., flowers) or vegetative organs (e.g., leaves, wood, bark) in several eudicot lineages (Metcalf & Chalk 1950; Gregory & Baas 1989; Matthews & Endress 2006), are interpreted as cells containing mainly polysaccharides, especially pectins (Fahn 1979; Matthews & Endress 2006), while the exudate of *Anulocaulis* is a mixture of different compounds. The presence of mucilage has been thought to be related to the ability of the plant to absorb and store water when available (Gregory & Baas 1989 and references therein). For a long time, this idea has been repeated for plants from arid and stressful environments, such as deserts (Gregory & Baas 1989), Mediterranean habitats (Christodoulakis et al. 1990) and sandy coastal areas (Ballejo-Campos & Paiva 2018). However, other authors have criticized this or proposed alternative interpretations such as the potential to reduce transpiration (Gregory & Baas 1989). It may also be a self-sealing mechanism, that is, to protect plants from dehydration and infections after

damage by sealing wounds (Anandan et al. 2018). In any case, it has been accepted that the capacity in storage and water retention of epidermal cells may be determined by the amount and composition of pectins in thickened walls (Voragen et al. 2009; Kuster et al. 2018), as seen in *Anulocaulis* and indicated for similar epidermal cells in secondary pollen presenting structures as observed in *Vangueria infausta* (Rubiaceae) (Tilney et al. 2014). Besides the similarity in shape, the epidermal cells in *V. infausta* were also reported with a cytoplasm rich in organelles, including secretory vesicles that might be involved in the production of hydrophilic and sticky substances (Tilney et al. 2014).

McClellan and Boecklen (1993) experimentally examined the role of the secretory rings in *Boerhavia spicata* and determined that such structures appear to discourage ant-aphid colonization or reduce ant or aphid density. According to the authors, the “sticky rings” work as traps, functioning in a way similar to other external secretory structures (e.g., glandular trichomes) or acting to diminish aphid populations. Indeed, this sticky plant defense syndrome, as reported for other groups, is likely to be an effective way for plants to cope with predators, especially those from arid and Mediterranean environments since their secretions are not as likely to be washed away in rainstorms (Karban et al. 2019). Moreover, the presence of phenolic compounds in the exudate of *Anulocaulis* may corroborate the results found by McClellan and Boecklen (1993), since phenolics are well-known for their potential to provide chemical defense against pathogen activity and herbivory (Evert 2006). In addition, our observation that the exudate remains sticky for a long period of time supports the idea that the rings may have adaptive importance in immobilizing potential herbivores, as demonstrated in other plant-insect interactions associated with sticky secretion (Monteiro & Macedo 2014; Krimel & Pearse 2013; LoPresti et al. 2018; Karban et al. 2019), although their somewhat inconsistent expression indicates that if the rings represent a defense mechanism it might be inducible rather than constitutive.

To the best of our knowledge there has been no report of similar epidermal cells as the one described for *Anulocaulis* for other Nyctaginaceae. Nevertheless, columnar parenchyma cells have been described for the anthocarp walls in other taxa of tribe Nyctagineae (i.e., *Acleisanthes*, *Boerhavia*, and *Mirabilis*) by Wilson & Spellenberg (1977). Despite their structural similarity with the secretory ring cells in *Anulocaulis* (both are elongated), the cells in the fruits are located in the subepidermal region, and so seem to belong to the cortex instead of the epidermis. Although no histochemical analyses were performed for the columnar parenchyma cells, the authors observed that there is a copious discharge of mucilage-like material when the fruits of *Boerhavia* and *Mirabilis* were placed in water. The presence of these elongated cells and mucilage-like content was discussed as possible mechanisms for water retention, epizoochory, germination or even carnivory (Wilson & Spellenberg 1977).

The occurrence of secretory rings in *Anulocaulis* and related genera have captured the attention of botanists at least since the segregation of the genus *Anulocaulis* by Standley (1909). More recently, in a morphological study to test the monophyly of *Anulocaulis* and related genera, including *Boerhavia* and *Cyphomeris*, Hernández-Ledesma et al. (2010) performed a cladistic analysis and concluded that the occurrence of the “mucilaginous rings” was not corroborated as a synapomorphy for these genera, indicating likely a convergence in the evolution of this character. Finally, there is variation in the degree to which the rings manifest within and among populations. It is unknown whether there is also variation between years. Thus, it is not clear whether they represent an inducible response to herbivory or other stimuli.

Although further experimental and ultrastructural analyses are required to elucidate the secretory activity and the mechanism of secretion, the findings obtained here shed light into the structure, development, and histochemistry of this secretory structure which was poorly known in the family. Indeed, more work is needed to compare the anatomy and chemical components of the secretory rings within related genera of Nyctaginaceae to search for potential homologies in other secretory ring-bearing species, or to the unusual viscid secretions found commonly on fruits of species in genera in different tribes of Nyctaginaceae (Wilson & Spellenberg 1977; Sukhorukov et al. in prep.). Finally, additional research into the ecology of this structure (its expression, and its effects on herbivores or seed predators) is needed to understand its function.

APPENDIX 1

Information on taxa, collectors, localities and herbarium vouchers for the analyzed species.

- Anulocaulis leiosolenus** var. **leiosolenus** (Torr.) Standl. USA. Texas. Sierra Blanca, Malone Mountains, 31.25342, -105.598, 13 Sep 2018, Douglas & Cunha Neto 2278 (BRIT, FLAS).
- Anulocaulis leiosolenus** var. **gypsoigenus** (Waterf.) Spellenb. & Wootten. USA. New Mexico, Eddy County, Yeso Hills, 32.018269, -104.433307, 13 Sep 2018, Douglas & Cunha Neto 2280 (BRIT, FLAS); Crest of 7 Rivers Hills, 32.487, -104.335, 14 Sep 2018, Douglas & Cunha Neto 2277 (BRIT, FLAS).
- Cyphomeris gypsophiloides** (M. Martens & Galeotti) Standl. USA. Las Cruces, New Mexico, Organ Mountains-Desert Peaks National Monument, 32.33642, -106.59743, 15 Sep 2018, Douglas et al. 2287 (BRIT, FLAS).

ACKNOWLEDGMENTS

This study was financed by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Proc. 2017/17107-3) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) – Finance Code 001. We thank Patrick Alexander (Bureau of Land Management, USA) for kindly helping with field work and the Laboratory of Plant Anatomy at the University of São Paulo (USP) for the use of their facilities for conducting the study, and Michael Moore and Richard Spellenberg for thoughtful comments that improved the manuscript.

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