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Distribution of cystoliths in the leaves of *Acanthaceae* and its effect on leaf surface anatomy

N.H. Gabel[1], R.R. Wise[1,*], G.K. Rogers[2]

Key words

Acanthaceae
calcium carbonate
cystolith
leaf epidermal impression
lithocyst

Abstract Cystoliths are large outgrowths of cell wall material and calcium carbonate with a silicon-containing stalk found in the leaves, stems and roots of only a handful of plant families. Each cystolith is contained within a cell called a lithocyst. In leaves, lithocysts may be found in the mesophyll or the epidermis. A study by Koch et al. (2009) reported unique, indented features on the surface of superamphiphilic *Ruellia devosiana* (*Acanthaceae*) leaves which the authors named 'channel cells'. We report herein that such 'channel cells' in the *Acanthaceae* are actually lithocysts containing fully formed cystoliths in which only a portion of the lithocyst is exposed at the epidermis, forming a leaf epidermal impression. Intact leaves and isolated cystoliths from 28 *Acanthaceae* species (five in the non-cystolith clade and 23 in the cystolith clade) were examined using light and scanning electron microscopy and X-ray microanalysis. All 23 members of the cystolith clade examined contained cystoliths within lithocysts, but not all showed leaf epidermal impressions. In four species, the lithocysts were in the leaf mesophyll, did not contact the leaf surface, and did not participate in leaf epidermal impression formation. The remaining 19 species had lithocysts in the epidermis and possessed leaf epidermal impressions of differing sizes, depths and morphologies.

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INTRODUCTION

Cystoliths (literally, 'cavity stone', Wedell 1854) are large growths of cellulosic cell wall material and amorphous calcium carbonate found in the leaves, stems and roots of only about eight of the 406 Angiosperm families. Cystoliths have been confirmed in the *Acanthaceae* (*Asterids*, *Lamiales*), *Boraginaceae* (*Asterids*, *Boraginales*), *Cannabaceae* (*Rosids*, *Rosales*), *Cucurbitaceae* (*Rosids*, *Cucurbitales*), *Moraceae* (*Rosids*, *Rosales*), *Opiliaceae* (*Rosids*, *Santales*), *Ulmaceae* (*Rosids*, *Rosales*) and *Urticaceae* (*Rosids*, *Rosales*) (Solereder 1908b, Linsbauer 1921, Metcalfe & Chalk 1950). Cystoliths are apoplastic; that is, they lie outside the plasma membrane and protrude into a single cell to the extent that the cell interior may be almost completely filled. The cell in which the cystolith is located is termed a lithocyst ('stone cavity') and the intrusive growth of the lithocyst is coordinated with the growth of the cystolith (Ajello 1941, Kuo-Huang & Yen 1996). When they occur in leaves, cystoliths may be found in either the epidermis or the mesophyll, as in the *Acanthaceae*, *Moraceae* and *Urticaceae*, or in trichomes as in the *Boraginaceae*, *Cannabaceae* and *Cucurbitaceae* (Solereder 1908a). [Note: The chemical composition and location of cystoliths clearly distinguish them from calcium oxalate (CaOx) crystals, a separate type of plant secretion, synthesized and located entirely within the cell vacuole. The biology of CaOx crystals has been extensively studied (Nakata 2003)].

Cystoliths receive little attention in the modern literature but have been known for over 160 years (Wedell 1854). The cystoliths of the *Acanthaceae* are particularly prominent and were studied extensively as potential taxonomic characters late in the 19th century by Hobein (1884). In fact, he was able to separate the family into two major groups based on the presence or absence of cystoliths. More recent molecular studies have recognized a 'cystolith clade' and a 'non-cystolith clade' (McDade et al. 2008) that correspond to and largely support Hobein's early classification system. The *Acanthaceae* is a family of 217 recognized genera and some 2 500 species (Scotland & Volleson 2000, McDade et al. 2008). Thirty-three genera (15 %) are placed in the non-cystolith clade with the remaining 183 (85 %) in the cystolith clade (one genus remains unplaced, *Dolichostachys* Benoist) (from Scotland & Volleson 2000, McDade et al. 2008).

In 2009, Koch et al. published a report on the wetting properties of *Ruellia devosiana* Hort.Makoy ex É.Morren leaves (*Acanthaceae*). Most notably, the leaves were found to be superamphiphilic. That is, the surfaces were both superhydrophilic and superoleophilic, an unusual combination of properties. In addition to the unique chemical properties, Koch et al. (2009) reported the presence of "unidirectional expanded cells with a flat periclinal wall". They named these 'channel cells', because they are slightly sunken below the surface and create channel-like structures in combination with the surrounding epidermal cells. However, they made no mention of cystoliths, which can be seen in their work and are known to be present in all *Ruellia* L. species (Hobein 1884).

Upon conducting an anatomical survey of species in the family *Acanthaceae*, we noted that Koch's 'channel cells' are to be found on leaves of many, but not all, members of the so-called *Acanthaceae* cystolith clade and always co-localize with cystoliths. Indeed, 'channel cells' are in fact epidermal lithocysts con-

[1] Department of Biology, University of Wisconsin Oshkosh, 800 Algoma Blvd., Oshkosh, WI 54901, USA; corresponding author email: wise@uwosh.edu.
[2] Department of Horticulture, Palm Beach State College, 3160 PGA Boulevard, Palm Beach Gardens, FL 33410, USA.

Table 1 Species examined, including presence or absence of channel cells and collection data.

Species	Leaf epidermal impressions	Collection site	Voucher
Non-cystolith clade			
Avicennia germinans (L.) L.	Absent	Martin Co. FL	OSH-124646
Crossandra infundibuliformis (L.) Nees	Absent	Palm Beach Co. FL	OSH-124629
Thunbergia alata Bojer ex Sims	Absent	Palm Beach Co. FL	OSH-124641
Thunbergia erecta T.Anderson	Absent	Palm Beach Co. FL	OSH-124640
Thunbergia grandiflora Roxb.	Absent	Palm Beach Co. FL	OSH-124639
Cystolith clade			
Asystasia gangetica (L.) T.Anderson	Common on both surfaces	Palm Beach Co. FL	OSH-124649
Barleria cristata L.	Uncommon, on adaxis only	Palm Beach Co. FL	OSH-124644
Barleria repens Nees	Common, on adaxis only	Palm Beach Co. FL	OSH-124634
Eranthemum pulchellum Andrews	Common on both surfaces	Palm Beach Co. FL	OSH-124648
Fittonia albivenis (Lindl. ex Veitch) Brummitt	Absent	Palm Beach Co. FL	OSH-124650
Graptophyllum pictum (L.) Griff.	Absent	Palm Beach Co. FL	OSH-124633
Hygrophila polysperma, emergent (Roxb.) T.Anderson	Common on both surfaces	Martin Co. FL	OSH-124647
Hygrophila polysperma, submerged	Absent	Martin Co. FL	OSH-124645
Hypoestes phyllostachya Baker	Common on both surfaces	Palm Beach Co. FL	OSH-122017
Justicia brandegeeana Wassh. & L.B.Sm.	Common on both surfaces	Palm Beach Co. FL	OSH-124632
Justicia spicigera Schltdl.	Common on both surfaces	Palm Beach Co. FL	OSH-124636
Megaskepasma erythrochlamys Lindau	Absent	Palm Beach Co. FL	OSH-124635
Odontonema tubaeforme (Bertol.) Kuntze	Common, on abaxis only	Palm Beach Co. FL	OSH-124637
Pachystachys lutea Nees	Common on both surfaces	Palm Beach Co. FL	OSH-124643
Pseuderanthemum carruthersii (Seem.) Guillaumin	Common on both surfaces	Palm Beach Co. FL	OSH-124642
Ruellia blechum L.	Common on both surfaces	Palm Beach Co. FL	OSH-124651
Ruellia caroliniensis (J.F.Gmel.) Steud.	Common on both surfaces	Palm Beach Co. FL	not vouchered
Ruellia humilis Nutt.	Common on both surfaces	Winnebago Co, WI	OSH-122089
Ruellia simplex C.Wright	Common on both surfaces	St. Lucie Co. FL	OSH-122017
Ruttya fruticosa Lindau	Common, on adaxis only	Palm Beach Co. FL	OSH-124628
Sanchezia speciosa Leonard	Absent	Palm Beach Co. FL	OSH-124630
Strobilanthes alternata (Burm.f.) Moylan ex J.R.I.Wood	Common on both surfaces	Palm Beach Co. FL	OSH-124631
Strobilanthes auriculata var. *dyeriana* (Mast.) J.R.I.Wood	Common on both surfaces	Palm Beach Co. FL	OSH-124627
Strobilanthes cusia (Nees) Kuntze	Common on both surfaces	St. Lucie Co. FL	OSH-124644

taining a prominent cystolith in which only a portion of the lithocyst wall is exposed at the leaf surface. We therefore prefer the name 'leaf epidermal impressions' to describe these striking features. Neither cystoliths nor leaf epidermal impressions are found in members of the non-cystolith *Acanthaceae* clade. Herein, we describe the anatomy and ultrastructure of the cystolith-containing epidermal lithocysts in a number of species in the *Acanthaceae* and note considerable variation between and among leaf epidermal impressions. Also, we discuss the history of the discovery of leaf epidermal impressions in the *Acanthaceae*. Our data enhance existing knowledge regarding the structural characteristics of leaf surfaces in the *Acanthaceae*.

MATERIALS AND METHODS

Source of study material

Most of the material was collected in Palm Beach County, Martin County or St Lucie County, Florida, largely from cultivated material. *Ruellia humilis* was collected in Winnebago County, Wisconsin. Leaves from at least three individuals of each species were examined. Voucher specimens and author citations for species names are in Table 1.

Leaf clearings

Fresh, unfixed leaves were frozen for 1–7 days and then cleared using the method of Vasco et al. (2014). Cleared material was dehydrated in ethanol, transitioned to 100 % xylene and mounted in Permount mounting medium (Fisher Scientific, Pittsburgh, Pennsylvania). Brightfield and polarized light microscopy were performed with an Olympus light microscope (LEEDS, Minneapolis, Minnesota). Images were captured digitally.

Fixation, dehydration, light and scanning electron microscopy

Samples for SEM microscopy were collected in the field and immediately fixed in FAA (3.9 % v/v formaldehyde, 47 % v/v ethanol, 2.5 % v/v glacial acetic acid). Fixation continued for 5–7 days. For light microscopy, fixed material was dehydrated through a graded alcohol series, embedded in Spurr's (1969) epoxy, sectioned and stained with toluidine blue (1 % in 1 % sodium borate). For scanning electron microscopy, fixation was followed by dehydration in ethanol and critical point drying in a Samdri-PVT-3D (Tousimis, Rockville, Maryland). Specimens were mounted to aluminium stubs using adhesive tabs and coated with gold/palladium in a Desk II sputter coater (Denton Vacuum, Moorestown, New Jersey). Digital images were acquired with a Hitachi S-3000N scanning electron microscope (Hitachi High Technologies America, Schaumburg, Illinois) at a variety of accelerating voltages and working distances.

To achieve SEM images of leaf cross sections, fixed material was dehydrated to 100 % ethanol as described above. Samples were then frozen in liquid nitrogen and fractured on a cold anvil with a cold razor blade. They were then thawed, critically point dried, mounted, coated and imaged in the SEM as above.

X-ray microanalysis

Samples were prepared as for SEM, see above, and analysed for chemical content using a Noran Voyager microanalysis system equipped with an energy dispersive spectrometer detector (Noran Instruments, Middleton, Wisconsin). Excitation voltage was 20 kV and a working distance of 20 mm was used.

Isolation of cystoliths

Cystoliths were isolated using the method of Maier & Arnott (2002). Fresh leaf material was fixed in 100 % ethanol for 1–7 days. The material was then homogenized for 5 min in 100 % ethanol in a blender at high speed. The resultant slurry was filtered through cheesecloth then decanted into a 15 cm diam watch glass placed on a nutating mixer set at a low speed. Cystoliths were recovered from the centre of the watch glass and washed twice using the same procedure. They were then allowed to air dry prior to mounting, coating with Au/Pd and microscopy.

Image processing

Some images were adjusted for brightness, contrast and/or colour balance using Adobe Photoshop (San Jose, California), release 21.1.1.

RESULTS

Leaves of *Acanthaceae* contain numerous cystoliths (Fig. 1a). The cystoliths in the species we examined are long (~100–120 µm) and narrow (~20–30 µm) with a larger, somewhat bulbous end tapering to a smaller, pointed end and a warty surface (Fig. 1b, c). Other shapes have been reported in other *Acanthaceae* genera not examined here but the blunt/tapered shape is the most common one seen in the literature (Solereder 1908a, Karlström 1978, Inamdar et al. 1990, Patil & Patil 2011) and in the species we examined. The cystoliths are birefringent and transparent (Fig. 1a, b). Double cystoliths are common in a few genera, most notably *Barleria* L. (Fig. 4, Hobein 1884) but also *Crabbea* Harv. and *Periblema* DC. (Hobein 1884).

Internally, the structure of the cystoliths we studied had concentric layers around an apparent core. Both the layers and core were visible in sectioned material for light microscopy (compare to Schacht 1855: f. 19) and fractured material for scanning electron microscopy (Fig. 2).

X-ray microanalysis was used to determine the chemical composition of cystoliths both *in situ* (Fig. 3) and isolated (not shown). *Acanthaceae* cystoliths are composed of calcium and oxygen, undoubtedly in the form of $CaCO_3$ (Schacht 1855) with a silicon-containing stalk. Carbon (not shown) was also present throughout, but it does not map well given that all the other structures in the leaf are carbon-based. The stalk presumably represents an attachment point between the cystolith and lithocyst; however, we were unable to visualize a physical attachment in any of the specimens we imaged.

All cystoliths, by definition, are located within a lithocyst (Wedell 1854) and, at maturity, the cystolith occupies the majority of the lithocyst volume (Fig. 4a). Lithocysts in the *Acanthaceae* species we studied had a nucleus, but very little cytoplasm (Fig. 4b).

The leaf epidermal impressions are prominent surface features of many, but not all, members of the *Acanthaceae* cystolith clade we examined (Fig. 5). They are found on the adaxial side, abaxial side or both surfaces in a species-specific manner (see Table 1). Other surface features include stomata (usually but not always restricted to the abaxial surface, Fig. 5c, e, f, h), glandular trichomes (Fig. 5a–c, g) and non-glandular trichomes (not shown). Biofilms were also commonly seen (Fig. 5h).

Leaf epidermal impressions are the exposed portion of cystolith-containing epidermal lithocysts (Fig. 6). Epidermal cystoliths in a

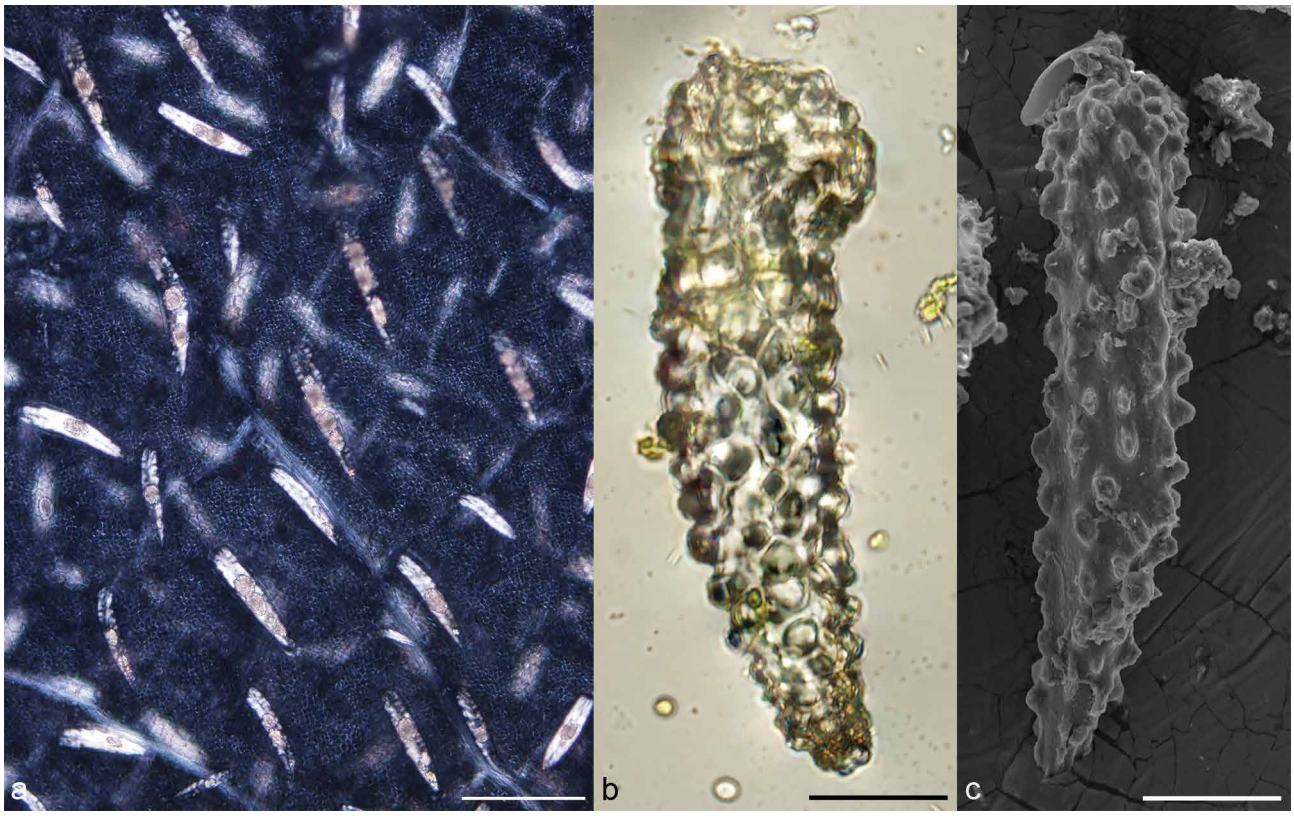

Fig. 1 *Acanthaceae* cystoliths are abundant. a. Light micrograph of cystoliths in a cleared *Ruellia humilis* leaf (OSH-122089) taken using cross-polarized microscopy. Note shape and birefringence of cystoliths; b. light micrograph of an isolated cystolith from *Barleria repens* (OSH-124634); c. SEM of an isolated *Eranthemum pulchellum* cystolith (OSH-124648). — Scale bars: a = 100 µM; b, c = 25 µm.

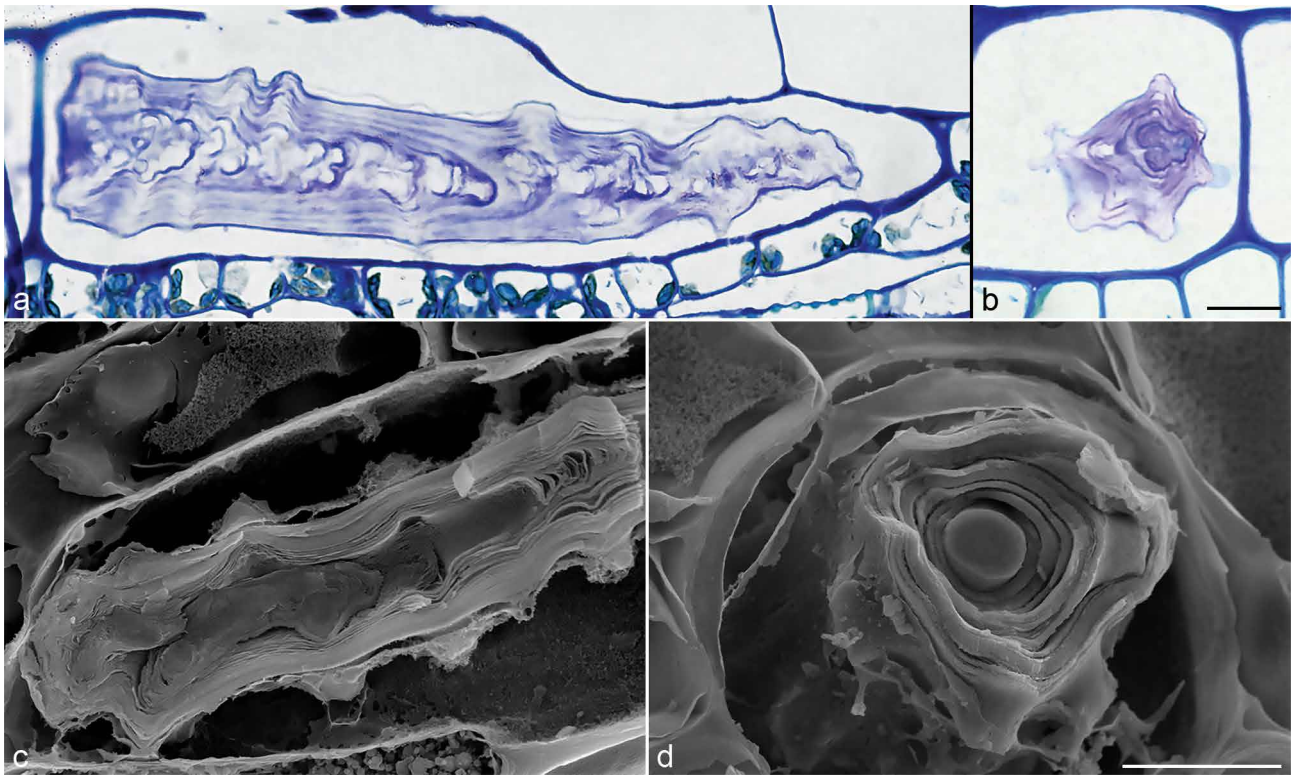

Fig. 2 Cystolith internal structure is layered around a central core. a. Longitudinal and; b. transverse sections of embedded material, *B. repens*; c. longitudinal and; d. transverse sections of fractured material of *Justicia spicigera* (OSH-124636). — Scale bars = 10 µm for all panels.

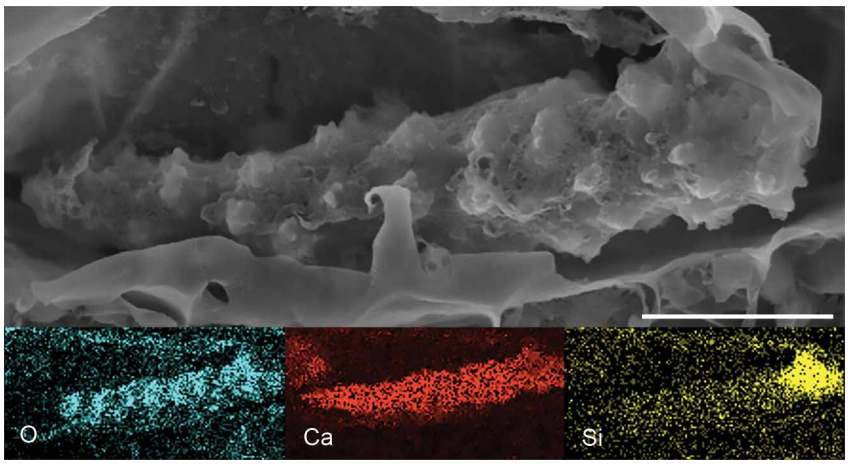

Fig. 3 Cystoliths are composed of calcium carbonate with a silicon-containing core. An SEM (top) and X-ray microanalysis maps (bottom) show the elemental composition of a *Ruellia simplex* (OSH-122017) cystolith. The entire cystolith contains $CaCO_3$ (oxygen in blue and calcium in red, carbon not shown) while silicon is only found in the bulbous end (yellow). — Scale bar = 25 µm.

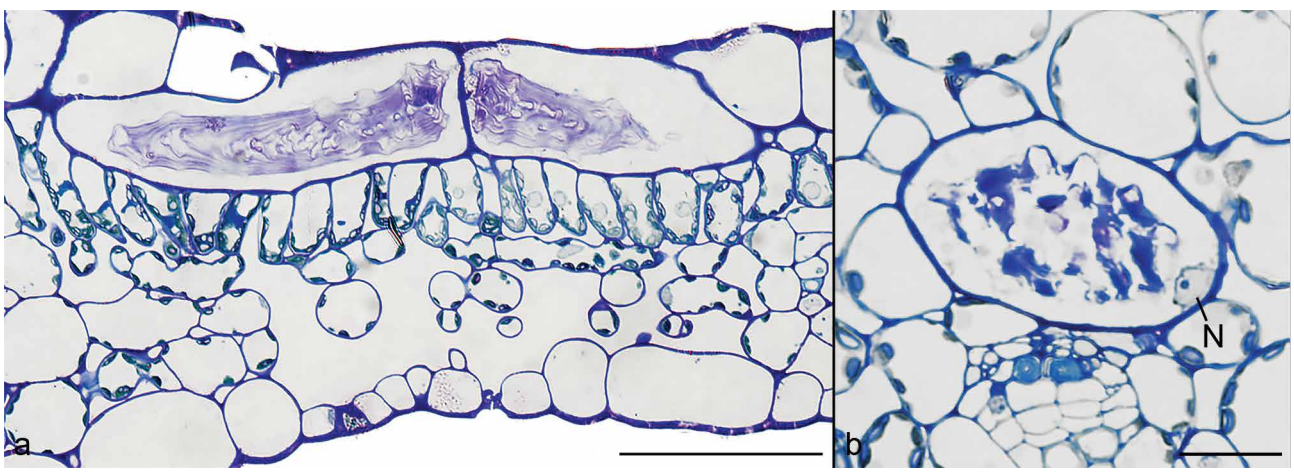

Fig. 4 Cystoliths are contained within a lithocyst. a. Two *Barleria repens* cystoliths in longitudinal section inside their respective lithocysts; b. *Barleria repens* lithocyst showing a cystolith in cross section and the cell's nucleus (N). Note paucity of cytoplasm. — Scale bars: a = 25 µm; b = 10 µm.

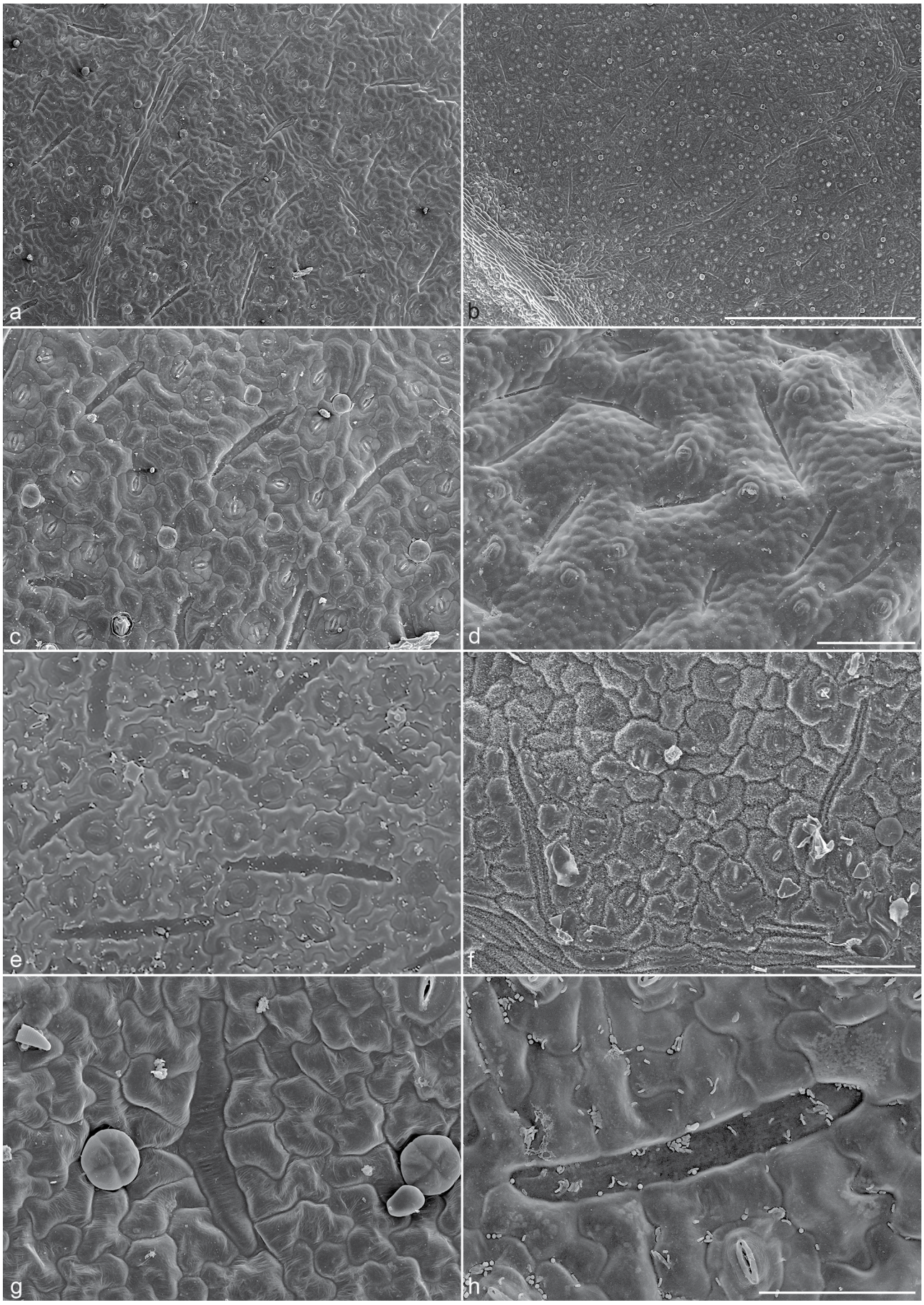

Fig. 5 Leaf epidermal impressions are diverse and abundant in some members of the *Acanthaceae*. a. *Ruellia simplex*; b. *Pachystachys lutea* (OSH-124643); c. *Ruellia simplex*; d. *Strobilanthes alternata* (OSH-124631); e. *Eranthemum pulchellum*; f. *Pseuderanthemum carruthersii* (OSH-124642); g. *Hygrophila polysperma* (emergent leaf) (OSH-124647); h. *Pachystachys lutea* (OSH-124643). — Scale bars: a, b = 1 mm; c, d = 200 µm; e, f = 100 µm; g, h = 50 µm.

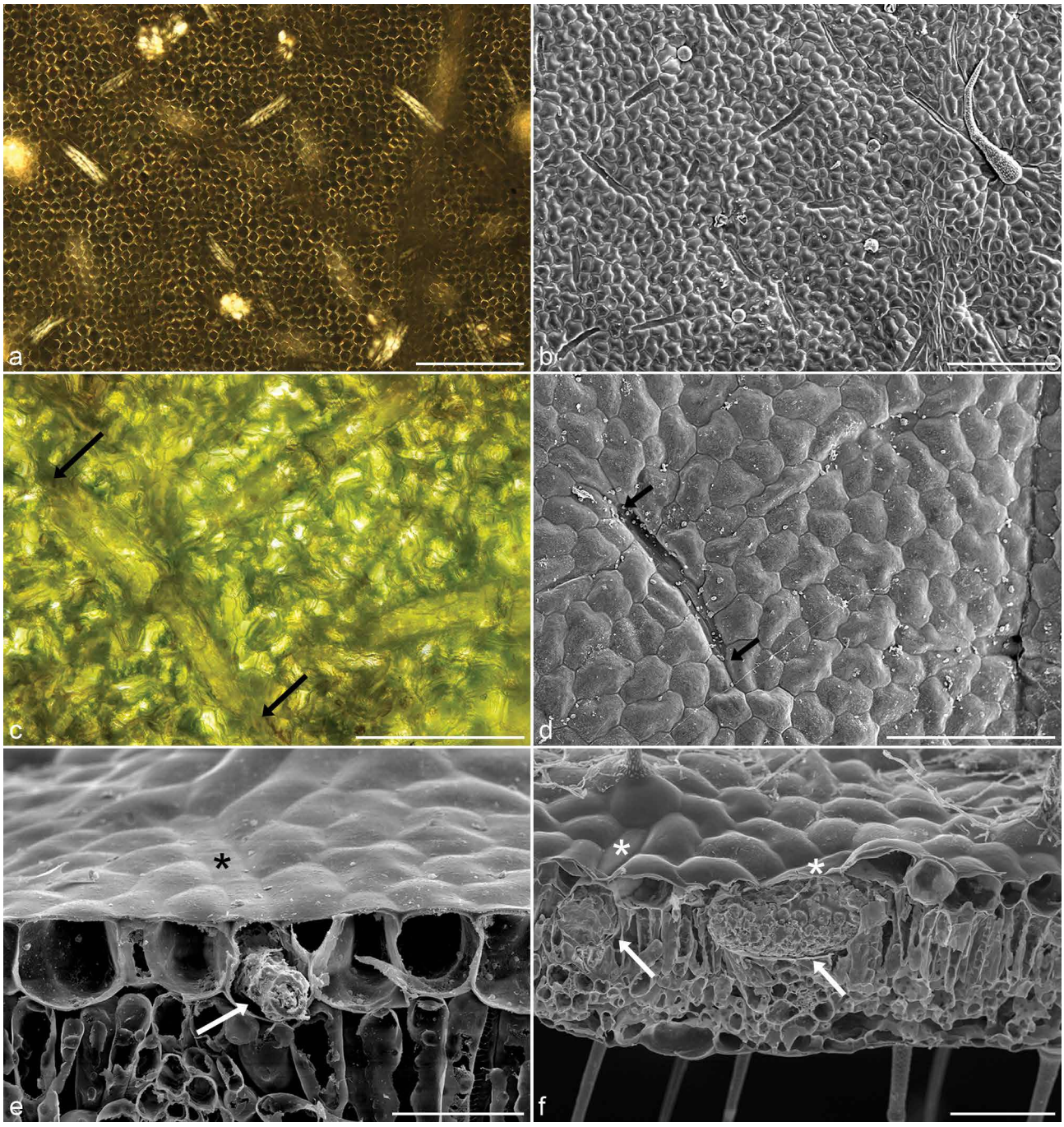

Fig. 6 Leaf epidermal impressions colocalize with cystolith-containing epidermal lithocysts. Correlative: a. LM (polarized) and b. SEM microscopy of the same *Pachystachys lutea* leaf taken at the same magnifications. Correlative: c. LM and d. SEM microscopy of the same *Barleria repens* leaf taken at the same magnifications. A cystolith pair in c and a leaf epidermal impression in d are indicated with arrows. Note a second cystolith pair and leaf epidermal impression in the upper right of the images. Leaf epidermises of e. *Strobilanthes alternata* (OSH-124631) and f. *Justicia brandegeeana* (OSH-124632); leaf epidermal impressions (asterisks), epidermal lithocysts (same asterisks) and cystoliths (arrows) in the adaxis. Note that the *S. alternata* cystolith is much smaller than seen in *J. brandegeeana*. — Scale bars: a–d = 200 µm; e = 50 µm; f = 100 µm.

Pachystachys lutea leaf, visible under polarized light, align with individual leaf epidermal impressions on the leaf surface seen in the SEM (Fig. 6a, b). Not all of the lithocyst participates in leaf epidermal impression structure. Figure 6c, d shows a pair of cystoliths and a leaf epidermal impression from a *Barleria repens* leaf. The double cystoliths are ~385 µm long while the leaf epidermal impression is only ~230 µm long. The majority of the cystolith is therefore subepidermal. Figure 6e, f directly shows the presence of a cystolith within an epidermal lithocyst. As noted above, not all of the lithocyst wall is exposed at the epidermis (cf. Fig. 4a, 6). The result of varying lengths of cystoliths and less than full participation of the lithocyst in leaf epidermal impression formation is that leaf epidermal impressions vary greatly in length. Figure 7 shows a short (~80 µm long) leaf epidermal impression on a *Justicia spicigera* leaf abaxial side and a long (> 600 µm) leaf epidermal impression on a *Strobilanthes cusia* leaf adaxis.

Lithocysts in *Graptophyllum pictum* (Fig. 8a) and *Megaskepasma erythrochlamys* (not shown) sit just under the epidermal layer and do not contact the leaf surface. Cystoliths in *Fittonia albivenis* leaves (Fig. 8b) are found in the leaf interior, near the boundary between the palisade and spongy mesophyll layers. The leaf mesophyll of *Sanchezia speciosa* has numerous cystoliths arranged both perpendicular and parallel to the leaf surface (Fig. 8c, d). All four species contain numerous cystoliths in their leaves, but none of them show leaf epidermal impressions on either the abaxial or adaxial leaf surfaces.

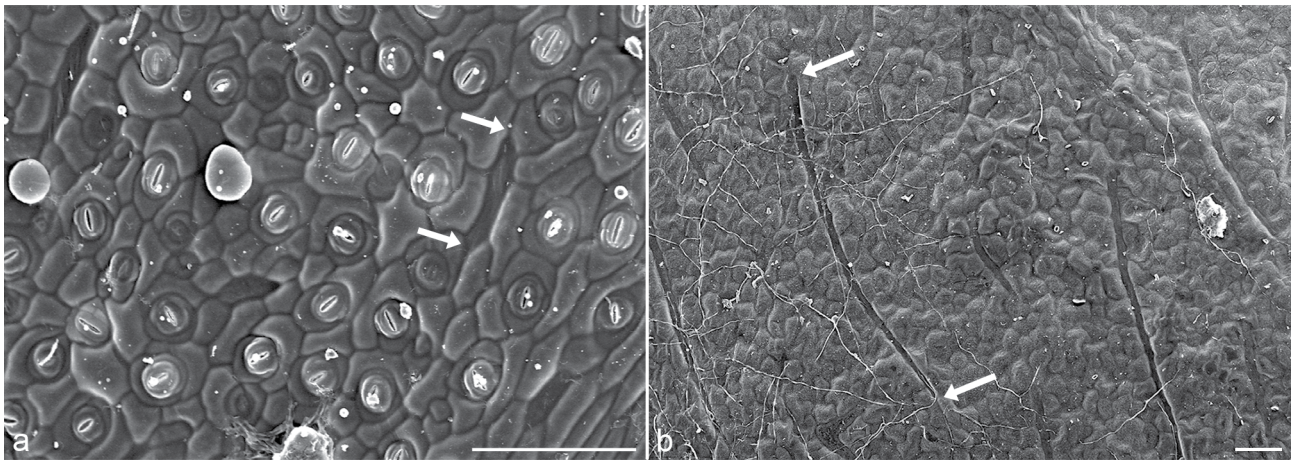

Fig. 7 Leaf epidermal impression length varies greatly. a. A leaf epidermal impression on a *Justicia spicigera* leaf (abaxial side) (OSH-124636) is approximately 80 µm long while that on; b. *Strobilanthes cusia* (adaxis) (OSH-124644) is over 600 µm long. Leaf epidermal impression ends are marked with arrows. — Scale bars = 100 µm in both panels.

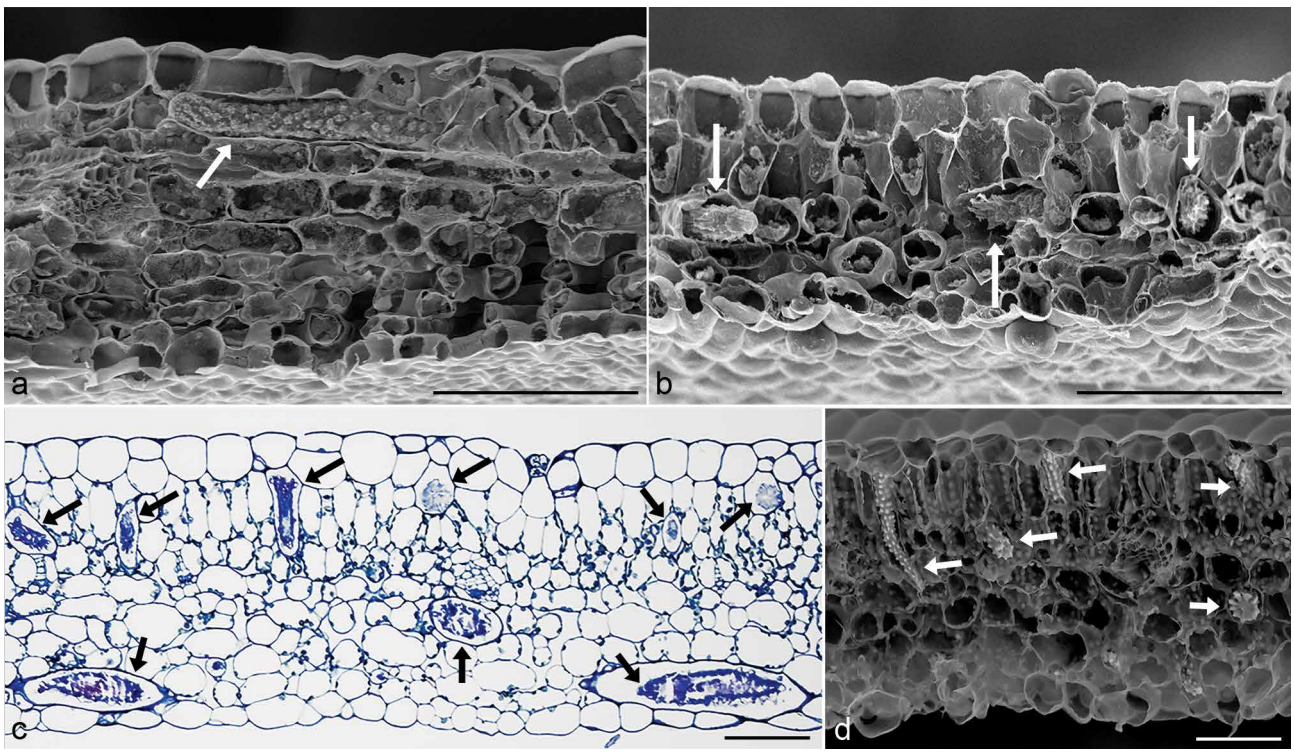

Fig. 8 Species with subepidermal lithocysts do not show leaf epidermal impressions. Cross sections of a. *Graptophyllum pictum* (OSH-124633) and; b. *Fittonia albivenis* (OSH-124650) leaves; c, d. cross sections of *Sanchezia speciosa* (OSH-124630) leaves. Nine cystoliths in c. and five in d. are in parallel or perpendicular orientation to the leaf surface. Cystoliths are indicated with arrows. — Scale bars: a, b = 100 µm; c, d = 200 µm.

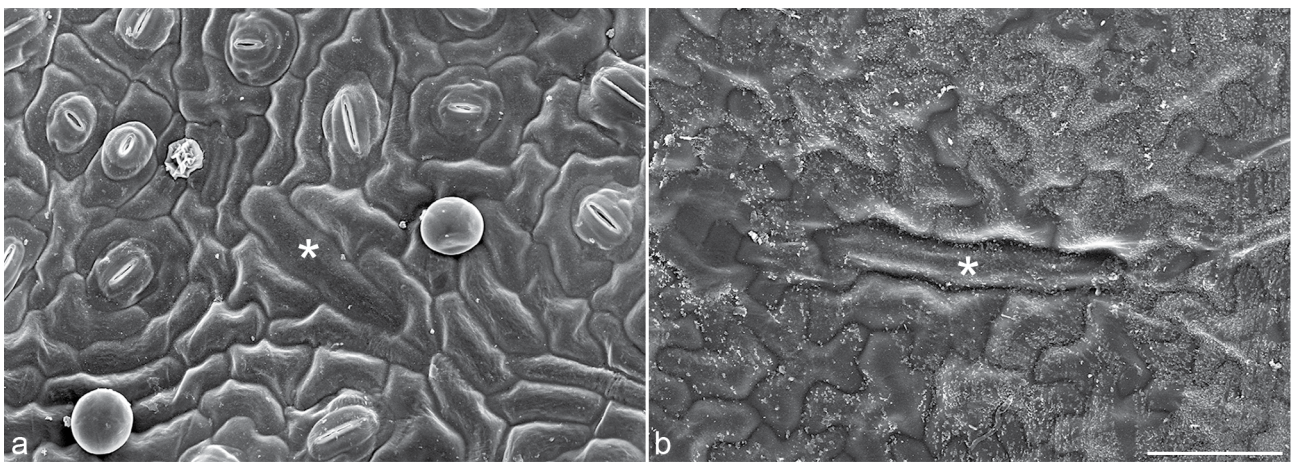

Fig. 9 An epidermal lithocyst may not necessarily form a (clear) leaf epidermal impression. a. *Strobilanthes auriculata* var. *dyeriana* (OSH-124627); b. *Ruttya fruticosa* (OSH-124628). Leaf epidermal impressions are indicated with asterisks (*). — Scale bar = 50 µm.

In another variation on lithocysts and leaf epidermal impressions, *Strobilanthes auriculata* var. *dyeriana* (Fig. 9a), *S. cusia* (not shown) and *Ruttya fruticosa* (Fig. 9b) possess epidermal lithocysts. However, the portion of the lithocyst that is exposed to the leaf surface does not make a leaf epidermal impression because the external lithocyst wall rises to the same level as the surrounding epidermal pavement cells.

DISCUSSION

Cystoliths in the *Acanthaceae* are primarily long and with either pointed or bulbous ends. Cystoliths found in other families can be similar or different in shape. The 'cavity stones' found in *Urticaceae* are similar with a taper (Watt et al. 1987) but the cystoliths found in *Moraceae* are round to pear shaped (Gal et al. 2012). Cystoliths in the *Cannabaceae* are teardrop-shaped and found in the base of trichomes (Dayanandan & Kaufman 1976).

Layering has been reported in the cystoliths of other species, but the acanth layers are thicker and fewer than those reported elsewhere. Gal et al. (2012) studied the internal structure of cystoliths from *Morus alba* L. and *Ficus microcarpa* L.f. While those structures also showed layering, the layers are more abundant and thinner in both species than in *Barleria repens*. Nitta et al. (2006) and Sugimura & Nitta (2007) also showed fine, thin layers in *M. alba* cystoliths as did Watt et al. (1987) in *Pilea cadierei* Gagnep. & Guillaumin (*Urticaceae*). X-ray microanalysis was unable to resolve if there are differences in elemental composition between the layers and the core (data not shown). While cystolith development has not been studied at the ultrastructural level in the *Acanthaceae*, presumably the layering relates to formation and growth.

Hobein (1884) and Solereder (1908b) reported that the cystolith stalk in the *Acanthaceae* probably dissolves at maturity, which is consistent with our inability to clearly visualize a stalk in any of the hundreds of individual specimens we examined. *Ficus elastica* Roxb. ex Hornem. (*Moraceae*) (Gal et al. 2012) and *Morus alba* (Nitta et al. 2006) also have a silicon-rich cystolith stalk; however, stalks in those genera persist into maturity and clearly show attachment of the cystolith to the lithocyst wall.

In *Pilea* Lindl. (*Urticaceae*; Watt et al. 1987), cystolith formation begins with a silicaceous outgrowth of the lithocyst cell wall that pushes the plasma membrane against the tonoplast. Both cystolith and peg remain surrounded by the lithocyst plasma membrane. Cell wall material is synthesized in the adjacent cells and transported to the lithocyst via Golgi vesicles while calcium salts are transported across the lithocyst membrane. As the *Pilea* cystolith enlarges, so does the lithocyst which grows intrusively through the adjacent mesophyll tissue. The lithocyst remains alive at maturity with the cytoplasm containing abundant mitochondria, endoplasmic reticulum and ribosomes and an elongate nucleus. Cystolith development has been less studied in the *Acanthaceae*, and not at all at the ultrastructural level. Kuo-Huang & Yen (1996) monitored numbers and sizes of abaxial and adaxial lithocysts during leaf expansion in *Justicia* L. but did not detail the development of individual cystoliths. Linsbauer (1921) focussed on the loss of calcium from cystoliths during aging and had little to say regarding early development.

Leaf epidermal impressions in the *Acanthaceae* vary in length from 75–275 µm in *Pseuderanthemum* Radlk. (Choopan & Grote 2015), up to 400 µm in *Justicia* (Kuo-Huang & Yen 1996) and > 600 µm in *Strobilanthes cusia* (Fig. 7). This variation in length is due to differences in the lengths of the cystoliths themselves, as well as differences in how much of the lithocyst is exposed to the leaf surface.

All cystoliths develop within a lithocyst and leaf epidermal impressions are the exposed portion of an epidermal lithocyst. However, not all lithocysts in the *Acanthaceae* take part in leaf epidermal impression formation because the relationship between cystolith, lithocyst and leaf epidermal impression varies between species (see Table 1). The five *Acanthaceae* species from the non-cystolith clade were confirmed to lack cystoliths and, likewise, do not have leaf epidermal impressions on either leaf surface (not shown). All 23 species examined in the cystolith clade were confirmed to possess cystoliths. Twelve have epidermal lithocysts and prominent leaf epidermal impressions on both leaf surfaces. Four, *Barleria cristata*, *Barleria repens*, *Odontonema tubaeforme* and *Ruttya fruticosa*, have leaf epidermal impressions on the adaxial side only. Four of the cystolith-bearing species in the cystolith clade lack leaf epidermal impressions.

Cystoliths in the *Acanthaceae* have been studied for 165 years (Schacht 1855) and have been reported in leaves (this study and references herein), stems (Remadevi et al. 2006, O'Neill 2010, Tripp & Fekadu 2014), roots (Jani & Rudrappa 2014), and wood (Ter Welle 1980, IAWA Committee 1989, Carlquist 2001). Being prominent features, they have formed the basis of various taxonomic treatments within the family (Hobein 1884, Solereder 1908b, Patil & Patil 2011), treatments that have held up fairly well in light of more modern investigations (Scotland & Volleson 2000, McDade et al. 2008). Hobein's (1884) phylogeny of the *Acanthaceae* used language such as "epidermal lithocyst of the round cystoliths usually comes to the surface of the leaf with only a small part" and "the entire lithocysts may be exposed at the surface." Figures 144D and F in Solereder (1908a: 615) indicate the same. However, the prominence of such features and the recognition that the exposed part might lie below the leaf surface (the 'channel cells' of Koch et al. 2009) or protrude above (our observations) only came recently.

The present study demonstrates the relationship between cystoliths, epidermal lithocysts and leaf epidermal impressions in leaves of members of the family *Acanthaceae*. Intriguingly, leaf epidermal impressions may be present in at least one other family. Groult (1999) used SEM to image leaf surfaces in *Pilea microphylla* (L.) Liebm. (*Urticaceae*). Figure 1.4 in that paper shows what appears to be a bona fide leaf epidermal impression, which the author described as "empreintes au sein des épidermes" ("imprints within the epidermis").

The long history of cystolith studies in the *Acanthaceae* (Hobein 1884, Solereder 1908a) and the prominence and abundance of leaf epidermal impressions in many species in the cystolith clade, beg this question, "Why were leaf epidermal impressions not described in detail when they were first reported?" We postulate it is because they are only readily apparent in the three-dimensional images that are generated with a scanning electron microscope. Light microscopy of fresh or fixed material merely looks right through them. Shrinkage of dried herbarium specimens obscures them in archived material. It took the development of the SEM, which was first commercially available in 1965 (McMullan 1995) to easily visualize them.

The function of leaf epidermal impressions, indeed of cystoliths themselves, remains somewhat of an enigma. For cystoliths, Chareyre (1884) and Freisleben (1933) showed evidence that they are deposits of excess calcium in leaves. Gal et al. (2012) provided support for a role in light scattering. Kai & Okazaki (2003) proposed a role as cellular pH stats during nitrite assimilation. Okamoto & Rodella (2006) found that silkworms preferred feeding on mulberry leaves (*Moraceae*) from cultivars with a lower density of cystoliths, supporting an antiherbivory function. It is possible that cystoliths serve one, or more, of those functions. For leaf epidermal impressions, Koch et al.

(2009) focused on the properties of superamphiphilic *Ruellia devosiana* É.Morrren leaf surfaces. They noted, but did not comment on, leaf epidermal impressions. It is not known if leaf epidermal impressions play a role in the unique wettability traits, or if other members of the *Acanthaceae* family (those with and without cystoliths and leaf epidermal impressions) have similar leaf surface characteristics.

CONCLUSIONS

Some members of the *Acanthaceae* cystolith-clade have long, indented furrows on the leaf surface, which we have described as 'leaf epidermal impressions'. Leaf epidermal impressions are the portion of an epidermal lithocyst that contacts the leaf surface, although most of the lithocyst cell may lie beneath the surface and not participate in leaf epidermal impression formation. They are found on the adaxial, abaxial or both leaf surfaces in an apparent species-specific fashion. No leaf epidermal impressions are seen in *Acanthaceae* species with lithocysts wholly within the leaf mesophyll or in species in the non-cystolith clade. The role of leaf epidermal impressions in leaf surface properties will require further research.

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