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Comparative anatomy of leaves of *Kalanchoe pinnata* and *K. crenata* in sun and shade conditions, as a support for their identification

Nattacha S. Moreira,¹ Luana Beatriz S. Nascimento,¹ Marcos Vinicius Leal-Costa,³ Eliana S. Tavares^{*,1,2}

¹Laboratório de Anatomia Vegetal, Departamento de Botânica, Instituto de Biologia, Universidade Federal do Rio de Janeiro, Brazil,

²Programa de Pós-graduação em Biotecnologia Vegetal, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Brazil,

³Instituto Federal de Educação, Ciência e Tecnologia Fluminense, Campus Centro, Brazil.

Abstract: *Kalanchoe pinnata* (Lam.) Pers. and *K. crenata* (Andrews) Haw., Crassulaceae, are popularly used in the treatment of many diseases. Their biological activities, such as anti-leishmaniasis and analgesic, can be useful in phytotherapy. Both species are often misidentified as the other, because of their similar popular uses and names, and the similar external morphology of the leaves. We investigated the existence of anatomical characters that will permit correct identification of the species grown in shade and in sun conditions. We also contribute with new observations on the leaf anatomy of *K. pinnata* and *K. crenata*. Fixed (FAA₇₀) leaves were used, and their sections were embedded in Leica historesin. Hydathodes were observed in both species, and for the first time were anatomically described in *K. crenata*. The species showed anatomical differences in relation to the presence of epidermal idioblasts only in *K. crenata*, the different pattern of distribution of subepidermal idioblasts, and the presence of leaf buds only in *K. pinnata*.

Introduction

The species of Crassulaceae are Crassulacean Acid Metabolism (CAM) plants. Unlike C₃ and C₄ plants, CAM plants assimilate atmospheric CO₂ into C₄ acids at night, and subsequently fix this CO₂ to the carbohydrate level during the following day (Cushman & Bohnert, 1997).

Two species of this family, *Kalanchoe pinnata* (Lam.) Pers. and *K. crenata* (Andrews) Haw., are popularly used to treat several diseases, including bronchitis and gastritis (Moreira, et al. 2002; Medeiros et al. 2004; Silva et al., 2006). Besides their common uses, these species share characteristics related to leaf morphology, including decussate, succulent, and glabrous leaves; ovate to elliptical leaf blades; and crenate margins (Hyakutake & Grotta, 1972; Anjoo & Kumar, 2010). Although *K. crenata* has simple leaves and *K. pinnata* has simple or compound ones, their leaves are very similar, especially when *K. pinnata* has only simple leaves. Because of their similarities, both species are known in Brazil as folha-da-costa, saião, and coirama (Brito & Brito, 1993; Medeiros et al. 2004; Silva et al., 2006; Joseph et al., 2011).

Several biological activities have been reported for *K. pinnata* and *K. crenata*, including anti-leishmaniasis (Muzitano et al., 2006), antinociceptive, anti-inflammatory, and antidiabetic (Ojewole, 2005) for *K. pinnata*, and analgesic and anticonvulsant (Nguelefack et al., 2006) for *K. crenata*. In both species, the leaf is the plant organ that is most used in folk medicine and in studies of biological activity.

Leaves are highly susceptible to environmental variations, mainly light intensity. Leaves developed under high light (sun leaves) are usually smaller and thicker, frequently have a higher density of stomata, a thicker epidermis and cuticle, and more developed mesophyll compared to leaves developed under low light (shade leaves) (Dickison, 2000; Schulze et al., 2002).

Knowledge of leaf anatomy is essential for the registration and quality control of herbal medicines (Anvisa RDC No. 48/2004). Plants used in herbal medicines may be subject to different degrees of shading during growth. Therefore, studies of medicinal plants grown under different light conditions are important to examine photomorphogenic changes that may cause problems with their identification (Milaneze-Gutierrez et

al., 2003).

Some anatomical studies have examined *K. pinnata* (Jain et al. 2008; Anjoo & Kumar, 2010; Leal-Costa et al. 2010) and *K. crenata* (Hyakutake & Grotta, 1972). However, these studies provided neither information about the influence of environmental light intensity on the leaf anatomy of these species, nor a detailed anatomical description of them. Therefore, in view of the potential for development of herbal medicines from *K. pinnata* and *K. crenata* leaves, and considering the difficulties in differentiating between them based on their external morphology during the vegetative stage, this study aimed to contribute to their anatomical description and to highlight distinguishing characters that are stable in different light conditions.

Material and Methods

Plant material

Specimens of *Kalanchoe pinnata* (Lam.) Pers. and *K. crenata* (Andrews) Haw., Crassulaceae, were obtained from the Botanical Garden of Rio de Janeiro. The voucher specimens were deposited at the Universidade Federal do Rio de Janeiro Herbarium (RFA37525 and RFA37524, respectively).

Cultivation conditions

Young plants (4-5 months) were obtained from the Botanical Garden of Rio de Janeiro. Six specimens of each species were planted in individual hard plastic pots, with the same substrate and watering routine. Three plants of each species were grown in sun and three in shade (under a tree). Photosynthetically active radiation (PAR) was measured monthly during the course of a year, on sunny days, with a PAR sensor coupled to an FMS2 Hansatech fluorometer (Hansatech Instruments Ltd., King's Lynn, UK). The PAR intensity ranged from 413.9 to 801.3 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for the sun plants, and from 12.8 to 19.9 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for the shade plants.

Leaf anatomy

Three simple leaves from the fourth node of different specimens were fixed in FAA₇₀ (Johansen, 1940). Leaf fragments were embedded in Leica Histo-resin[®] and sectioned in a Spencer rotary microtome. Cross sections were made in the proximal, middle, and distal regions of the petiole, and in the base, middle-third and apex of the leaf blade. The sections were stained with toluidine blue and mounted in Entellan[®]. Paradermal sections were cut mechanically and the fragments were stained in hydroalcoholic safranin (Johansen, 1940). Microchemical tests were performed on fresh material:

Sudan III to reveal lipids (Sass, 1951), lugol for starch (Johansen, 1940), Coomassie brilliant blue for protein (Fisher, 1968), and potassium dichromate for phenolic compounds (Gabe, 1968). Measurements of epidermis and mesophyll thickness, number of vascular bundles over 1 mm, and stomatal density were made in the middle third of fourth-node leaves with an optical microscope (Zeiss Standard) equipped with a drawing tube. Three repetitions of ten measurements were made on leaves of different specimens. Statistical analysis was performed by the GraphPad InStat 3.0 for Windows[®] program, using the t test ($p < 0.05$). The photographs were taken by means of an Olympus CH30 light microscope with an attached Olympus PM-C35B camera.

Results and Discussion

Morphological differences between sun and shade plants

Kalanchoe pinnata (Lam.) Pers. and *K. crenata* (Andrews) Haw., Crassulaceae, plants grown in sun were taller, although sun plants are usually taller when grown in shade (Taiz & Zeiger, 2009). In both species, the sun plants had larger and thicker leaves than the shade ones (Figure 1). This result differs from observations reported for sun leaves regarding leaf size, which are generally smaller, but not for leaf thickness (Esau, 1974). *Kalanchoe crenata* showed rounded leaves with slightly crenate margins in shade conditions, and elliptical leaves with pronounced crenate margins in sun conditions. *K. pinnata* showed a purple coloration on the petiole and leaf blade margins only in sun conditions (Figure 1).



Figure 1. *Kalanchoe pinnata* grown under sun (1a) and shade (1b); *Kalanchoe crenata* grown under sun (1c) and shade (1d).

The plant height may have different patterns of response according to the species' adaptive capacity to changes in light intensity (Muroya et al., 1997). Responses in leaf size may also differ between shade-tolerant and intolerant species (Evans & Hughes, 1961; Dengler, 1980; Dale, 1988).

Leaf anatomy

In *K. pinnata* epidermis, the anticlinal walls were sinuous on both sides, but the sinuosity was more

pronounced on the adaxial surface (Figure 2a) than on the abaxial one, in sun leaves (Figure 2b). In *K. crenata* sun leaves, the anticlinal walls were straight to slightly sinuous on the adaxial surface (Figure 3a) and sinuous on the abaxial surface (Figure 3b). In shade leaves of both species, the degree of sinuosity was the same on both surfaces (Figures 2c-d, 3c-d).

Both species are amphistomatic with anisocytic stomata (Figures 2c, 3a). This state has also been reported for other species of Crassulaceae (Duarte & Zaneti, 2002; Chernetsky & Weryszko-Chmielewska, 2008).

Kalanchoe pinnata and *K. crenata* have fully formed functional stomata, as well as others that are still differentiating (Figures 2d, 3b). The same trait

was observed in *Kalanchoe pumila* by Chernetsky & Weryszko-Chmielewska (2008), and is related to the ability of leaves of succulents to undergo cell division long after the leaves are photosynthetically active (Ting & Gibbs, 1982).

In both species and cultivation conditions, the abaxial surface had more stomata than the adaxial one. The stomatal density was lower in shade plants (Table 1). Jain et al. (2008) found a stomatal density of 18-20 stomata/mm² in *K. pinnata*, but the light conditions were not described. According to Ting & Gibbs (1982), CAM plants commonly have stomatal densities varying from 10 to 65 mm⁻², as found in this study.

In both species, the epidermis in cross-section

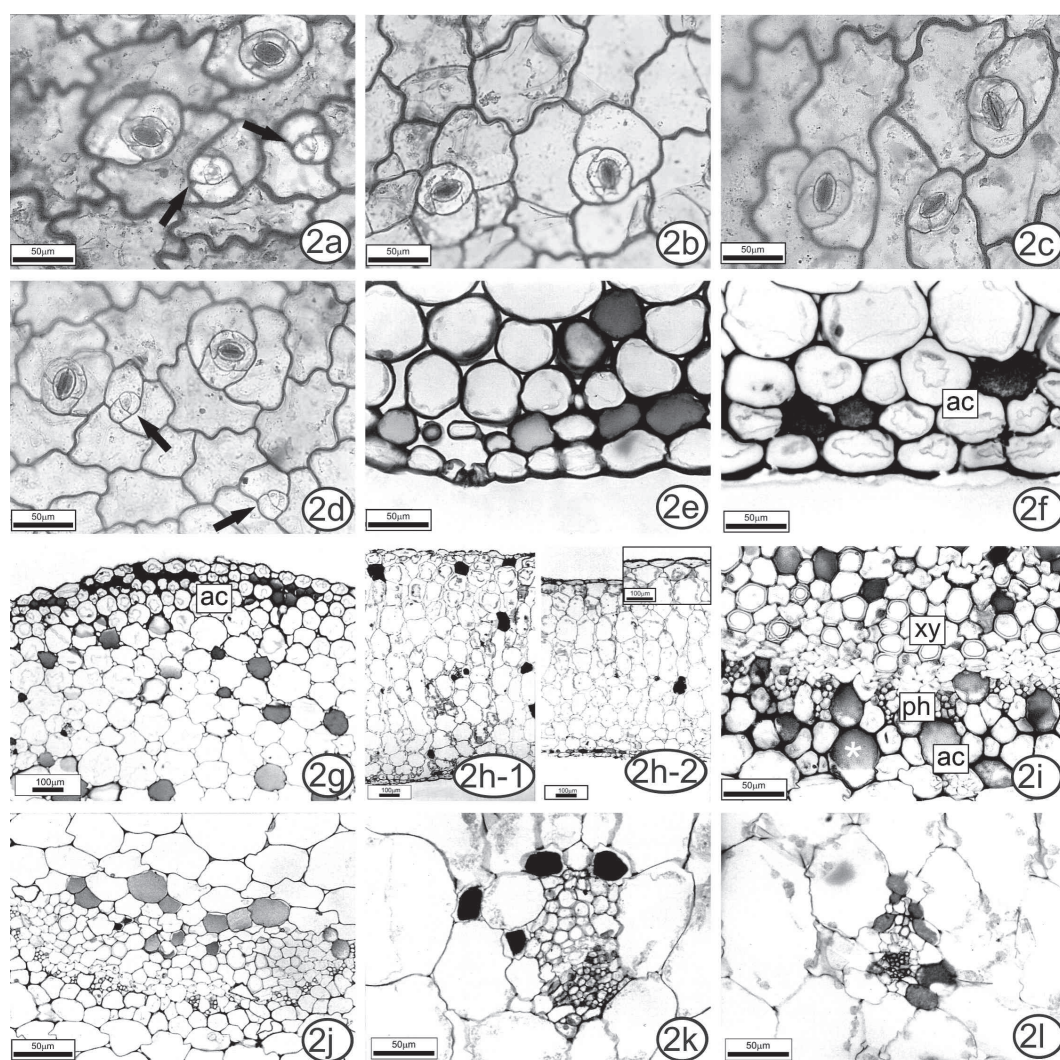


Figure 2. Aspects of the leaf anatomy of *Kalanchoe pinnata* grown in sun and shade. Paradermal sections: intercostal region of blade (a-b) in sun leaves; (c-d) in shade leaves. Anisocytic stomata on both surfaces and in different levels of differentiation (a,d - arrows). Cross sections of petiole (e,g) and blade (f,h-1): angular collenchyma in subepidermal position (f-g). Homogeneous mesophyll in sun plants (h-1) and shade plants (h-2). Collateral vascular bundles (i-l) more developed in sun plants (i,k) than in shade plants (j,l). Phenolic idioblasts (*). Legend: ac: angular collenchyma; xy: xylem; ph: phloem.

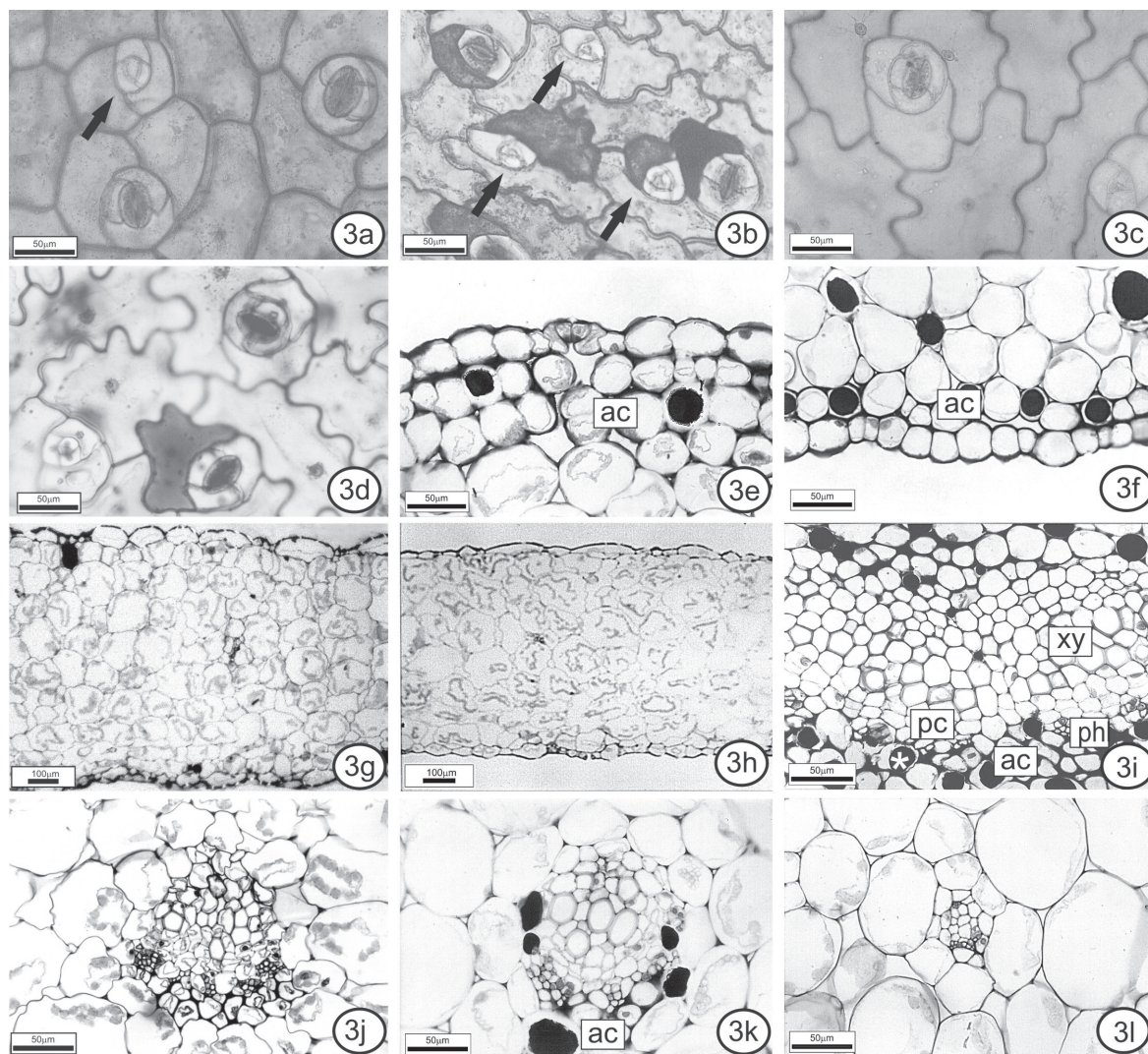


Figure 3. Aspects of the leaf anatomy of *Kalanchoe crenata* grown in sun and shade. Paradermal sections: intercostal region of blade (a-b) in sun leaves; (c-d) in shade leaves. Anisocytic stomata occur on both surfaces and in different levels of differentiation (a,b - arrows). Cross sections of petiole (e) and blade (f-l): angular collenchyma in the subepidermal position (e-f). Homogeneous mesophyll in sun plants (g) and shade plants (h). Collateral vascular bundles (i-l) more developed in sun plants (i,k) than in shade plants (j,l). Phenolic idioblasts*. Legend: ac: angular collenchyma; xy: xylem; ph: phloem.

Table 1. Comparison of anatomical characters of *Kalanchoe pinnata* and *Kalanchoe crenata* sun and shade plants.

	Stomatal density (stomata mm ⁻²)		Thickness of epidermis (μm)		Thickness of mesophyll (μm)	Number of vascular bundles
	Adaxial surface	Abaxial surface	Adaxial surface	Abaxial surface		
<i>K. pinnata</i> Sun	23.33±2.78 ^{A,a}	56.63±6.67 ^{B,a}	37.23±6.57 ^{A,a}	28.47±6.57 ^{B,a}	1264.23±21.8 ^a	1.77±0.63 ^a
<i>K. pinnata</i> Shade	17.80±5.06 ^{A,b}	40.93±5.17 ^{B,b}	29.93±5.84 ^{A,b}	24.09±7.30 ^{B,b}	766.42±78.10 ^b	1.43±0.57 ^b
<i>K. crenata</i> Sun	23.93±7.25 ^{A,a}	41.16±6.64 ^{B,a}	39.41±13.14 ^{A,a}	28.47±8.76 ^{B,b}	686.13±58.39 ^a	1.70±0.70 ^a
<i>K. crenata</i> Shade	15.70±3.97 ^{A,b}	30.63±8.22 ^{B,b}	38.67±9.49 ^{A,a}	28.47±7.30 ^{B,b}	577.37±42.34 ^b	1.30±0.50 ^b

*Different letters indicate significantly different values ($p \leq 0.05$, $n = 3$). For each parameter, capital letters indicate a comparison of values between the adaxial and abaxial surfaces; lower-case letters indicate a comparison between the values of sun and shade plants.

was uniseriate, with stomata at the same or slightly above the level of other epidermal cells (Figures 2e, 3e). These characteristics are shared by other crassulaceans (Duarte & Zanetti, 2002; Chernetsky & Weryszko-Chmielewska, 2008), except *Kalanchoe daigrementiana*, which has one to three epidermal cell layers (Balsamo & Uribe, 1988).

Epidermal cells of the petiole varied little in shape and size (Figures 2e, 3e). In the leaf blade, the cells were more rounded in the midrib (Figures 2f, 3f), and rectangular-flat in the intercostal region (Figures 2h, 3g-h). The epidermal cells of the leaf margin were larger than in the other regions (Figures 4a, c). The epidermis was covered by a thin cuticle.

K. pinnata sun plants had a thicker epidermis

than shade plants (Table 1), a feature generally described (Schulze et al., 2002). *Kalanchoe crenata* sun and shade plants did not show significant differences in epidermal thickness (Table 1). In both species and both cultivation conditions, the epidermal cells were thicker on the adaxial surface.

In both species, sun plants had some angular collenchyma layers in the subepidermal position, in the petiole (Figures. 2g, 3e), and in the blade midrib (Figures 2f, 3f). In shade plants, this tissue was less developed, with a more restricted distribution. Below the collenchyma, some chlorenchyma layers followed by ground parenchyma occurred.

The mesophyll in both species was homogeneous

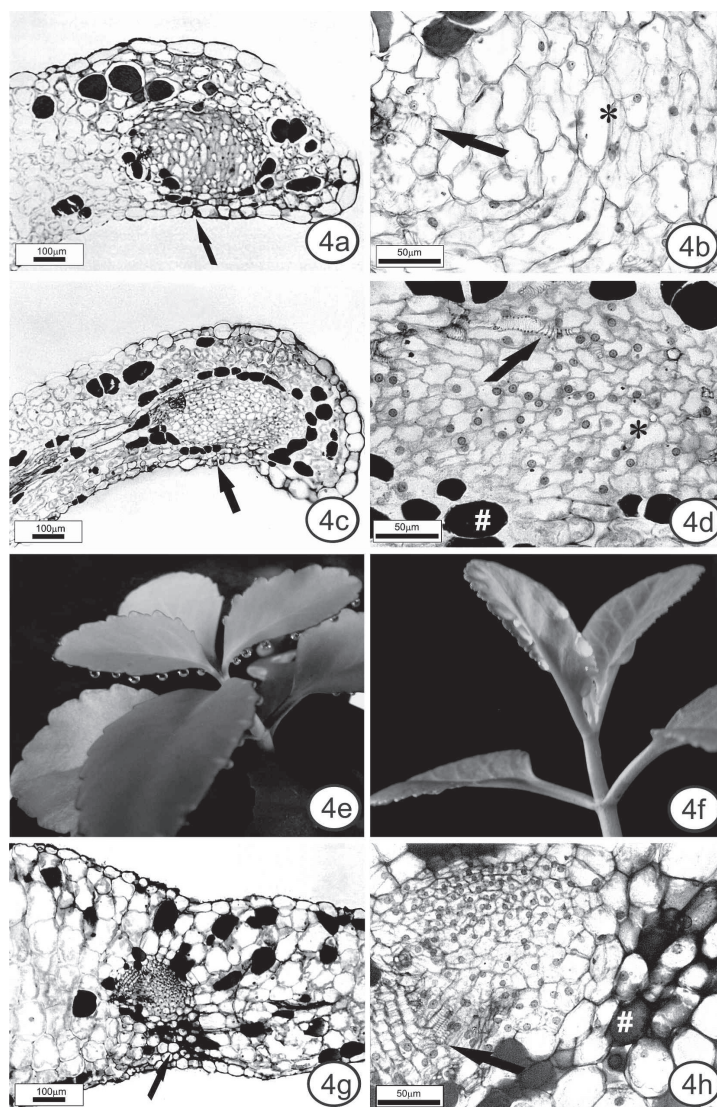


Figure 4. Anatomical aspects of the hydathodes and buds. Leaf cross sections of the hydathodes in *K. pinnata* (a,b) and *K. crenata* (c,d). Tracheids (b,d - arrows) reaching the epithem (*), and a stoma located on the abaxial side (a,c - arrows). Guttation under experimental conditions in *K. pinnata* (e) and *K. crenata* (f). Leaf cross sections of the structure of the buds in *K. pinnata* (g,h). Bud cells protected by collenchyma on the abaxial surface (g - arrow). Vascular bundle associated with the bud (h - arrow). Phenolic idioblasts (#) are related to hydathodes (d) and buds (h).

and the mesophyll thickness was significantly greater in sun plants (Table 1), because of a larger number of chlorenchyma layers (Figures 2h, 3g-h). The species shared some features that are commonly found in CAM species: succulent leaves, thick mesophyll with large cells with large vacuoles, and relatively small intercellular spaces (Nelson et al., 2005).

K. pinnata and *K. crenata* had collateral vascular bundles (Figures 2i-l, 3i-l), as observed for other crassulaceans (Duarte & Zaneti, 2002; Chernetskyy & Weryszko-Chmielewska, 2008). In the petiole and leaf midrib, the main vascular bundles had adjacent angular

collenchyma (Figures 2i-j, 3i-j). Small vascular bundles frequently had associated angular collenchyma in sun plants (Figure 3k). In shade plants, the vascular bundles were fewer (Table 1) and less developed (Figures 2l, 3l).

Hydathodes were observed in leaf blade margins in both species. In cross-section, they are delimited by a sheath (Figures 4a, c). The epithem is composed of various shapes and sizes of cells in *K. pinnata* and generally isodiametric cells in *K. crenata*, without obvious intercellular spaces (Figures 4b, d). Nuclei can be seen by light microscopy. Tracheids are seen between epithem cells (Figure 4b, d). A stoma is located on the abaxial

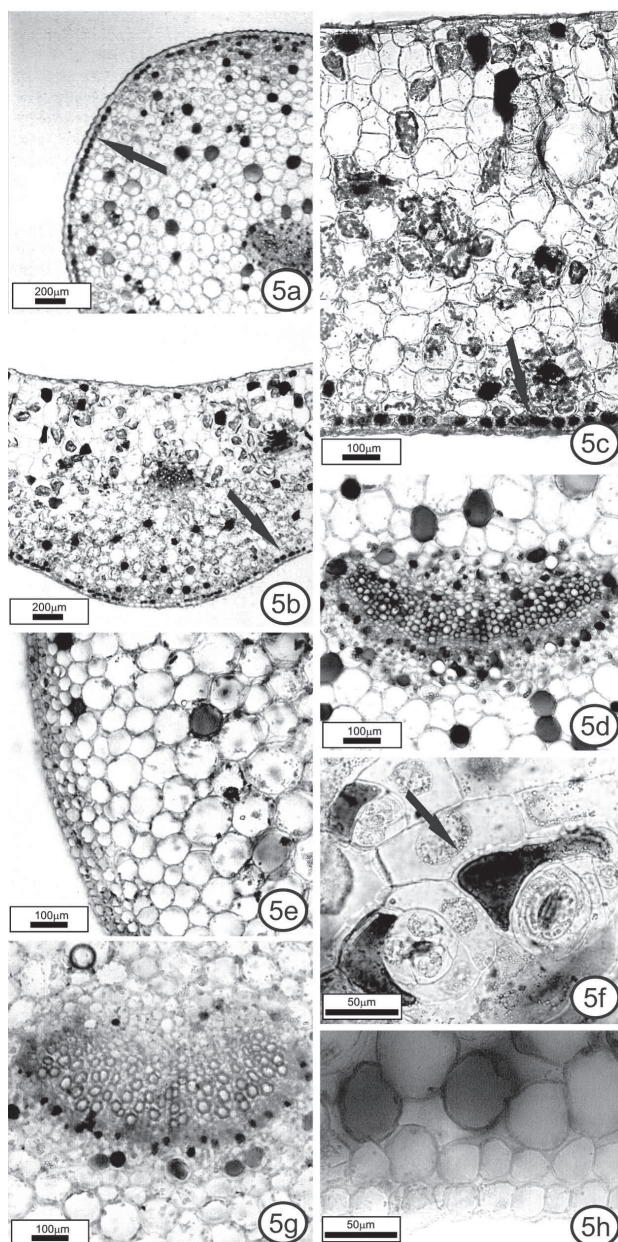


Figure 5. Microchemical test. Phenolic idioblasts in *K. pinnata* (a-d) and *K. crenata* (e-h). Idioblasts in the petiole (a,e,g) and leaf blade (b,c,d,f,h). In *K. pinnata*, the idioblast subepidermal layer can be observed, mainly in the petiole (a - arrow) and the midrib (b - arrow). In the midrib and the intercostal region (c), idioblasts were more numerous near the abaxial side. Only *K. crenata* showed idioblasts in the epidermis in the intercostal region (f - arrow).

surface (Figure 4a, c).

The hydathodes of *K. pinnata* and *K. crenata* differ from hydathodes of other families in having few intercellular spaces between epithem cells. There are no reports of hydathodes for the other species of *Kalanchoe*. The hydathodes are often related to guttation, and this phenomenon was observed in *K. pinnata* and *K. crenata* in experimental conditions (glass cover - Figure 4e, f).

The buds of *K. pinnata* are located in the deeper recesses of the leaf margin. They contain small meristem cells with visible nuclei and more differentiated isodiametric cells, some of them without an obvious nucleus. These cells are protected by collenchyma on the abaxial side (Figure 4g). There is also a vascular bundle associated with the bud (Figure 4h). According to Yarbrough (1932), as the leaf expands there is a tendency for a small group of marginal meristem cells to be set off or left behind in the development of the notch.

Microchemical tests

Phenolic idioblasts, revealed by the potassium dichromate and toluidine blue dye, were found along the entire petiole (Figure 5a, e, g) and leaf blade (Figure 5b-d, f, h), including close to hydathodes (Figure 4d) and buds (Figure 4h), in sun and shade *K. pinnata* and *K. crenata* plants.

K. pinnata contains large numbers of phenolic idioblasts in the subepidermal position, forming a layer in many regions, except at the leaf margin. The layers were observed mainly in the petiole (Figure 5a) and in the midrib (Figure 5b); in the midrib and in the intercostal region (Figure 5c), the idioblasts were more numerous near the abaxial side. A similar pattern of distribution was found in *K. pumila* (Chernetsky & Weryszko-Chmielewska, 2008). In *K. crenata*, the subepidermal idioblasts occurred isolated or together, but never forming a layer. Only *K. crenata* had idioblasts in the epidermis, in the intercostal region. They were more numerous on the abaxial surface (Figure 5f).

In shade plants of both species, the idioblasts showed the same pattern as sun plants, although they were less frequent. In a previous study, the production of phenolic compounds increased in *K. pinnata* grown *in vitro* under supplemental blue light (Leal-Costa et al., 2010).

Conclusion

In spite of the anatomical similarities of these species, differences were observed in the pattern of distribution of subepidermal idioblasts, which were isolated or grouped in *K. crenata*, but never formed layers as in *K. pinnata*; the occurrence of idioblasts in the epidermis only in *K. crenata*; and the presence of buds

only on the *K. pinnata* leaf margin. These differences remained constant under different lighting conditions. Therefore, they can aid in anatomy-level identification of the species and consequently in quality control of *K. pinnata* and *K. crenata* herbal medicines.

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***Correspondence**

Eliana S. Tavares
Laboratório de Anatomia Vegetal, Departamento de Botânica,
Instituto de Biologia, Universidade Federal do Rio de Janeiro
Avenida Professor Rodolpho Paulo Rocco s/n, Ilha do Fundão,
21.941-902 Rio de Janeiro-RJ, Brazil
elianast@biologia.ufrj.br
Tel. +55 21 2562 6328