Revista Brasileira de Farmacognosia 28 (2018) 9–15

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Original Article

Anatomy and microscopy of *Piper caldense*, a folk medicinal plant from Brazil

Vera Lucia P. dos Santos[a], Vijayasankar Raman[b], Vanessa B. Bobek[c], Izabel P. Migacz[c], Célia Regina C. Franco[d], Ikhlas A. Khan[b], Jane M. Budel[c,*]

[a] *Escola Superior de Saúde, Biociência, Meio Ambiente e Humanidades, Centro Universitário Internacional Uninter, Curitiba, Paraná, Brazil*
[b] *National Center for Natural Products Research, School of Pharmacy, University of Mississippi, University, Oxford, MS, USA*
[c] *Programa de Pós-graduação em Ciências Farmacêuticas, Universidade Estadual de Ponta Grossa, Ponta Grossa, Paraná, Brazil*
[d] *Departamento de Biologia Celular, Universidade Federal do Paraná, Curitiba, Paraná, Brazil*

ARTICLE INFO

Article history:
Received 27 September 2017
Accepted 25 November 2017
Available online 15 December 2017

Keywords:
Anatomy
Pimenta-d'água
Piperaceae
Light and scanning electron microscopy

ABSTRACT

Piper caldense C. DC., Piperaceae, commonly known as "pimenta-d'água", "pimenta-darda" or "paguarandy" in Brazil, is a shrub that grows mainly in humid and shaded habitats. The present study investigates the anatomy of the leaves and stems of *P. caldense* by light and scanning electron microscopy in order to provide supporting data for correct identification of the species. The leaves are hypostomatic, have a 2-layered hypodermis, and posses pearl glands. The midrib shows a 'U'-shaped stele comprised of about ten collateral vascular bundles. The main anatomical marker of the stem is the presence of a continuous sclerenchymatous sheath in the pith. Two forms of calcium oxalate crystals, namely crystal sand and raphides, are observed in this species.

Introduction

Piper L. is the largest and the most representative genus of the family Piperaceae with about 290 species and 45 varieties occurring in Brazil (Guimarães et al., 2015). Several species of *Piper* are volatile oil producers and many of them are used in folk medicine. Biological activities have been reported for many species (Marques et al., 2010; Carrara et al., 2012; Marques and Kaplan, 2015; Santos et al., 2016; Silva et al., 2016). The taxonomy and delimitation of the genus are problematic because different species have very similar morphologies, making their morphological identification difficult. In that sense, anatomical studies are essential to provide additional data for solving taxonomic problems (Gogosz et al., 2012).

Piper caldense C. DC., commonly called "pimenta-d'água", "pimenta- darda" and "paguarandy" in Brazil, is found in humid and shaded environments. It is a shrub, reaching up to 3 m high, with smooth and glabrous stems. The leaves are alternate, petiolate, narrowly elliptic or oblong in shape, acuminate to cuspidate at apex, unequal to cordate at base, and entire along margins. The veins are pinnate, sulcate above and raised beneath. The inflorescences are spikes, solitary, borne opposite to the leaves, and pendulous (Sarnaglia-Junior et al., 2014).

In Brazil, *P. caldense* is used in folk medicine as a sedative, antidote for snake bites and for relieving toothaches (Cardozo Júnior and Chaves, 2003). The plants have been reported to exhibit antifungal (Cardozo Júnior and Chaves, 2003), antimicrobial (Alves et al., 2016; Freitas et al., 2016), acaricidal (Araújo et al., 2012) and molluscicidal (Takahashi et al., 2013) activities. Besides that, the hydroalcoholic extract of leaves have decreased alcohol consumption in rats (Pereira et al., 2015).

The essential oils have shown α-cardinal, α-muurolol, tujopsan-2-β-ol and δ-cadiene from the leaves, terpine-4-ol, α-terpineol, α-cadinol 2-β-ol from the stems, and valencene, pentadecane, elina-3,7-11-die-no α-terpineol from the roots as the majority compounds (Rocha et al., 2016).

Considering the morphological similarities among different species of *Piper*, and the fact that no previous work has studied the anatomy of *P. caldense*, the present study aims to investigate the anatomical features of the leaf and stem of the species in order to assist in the identification of the plant materials.

* Corresponding author.
E-mail: jane@uepg.br (J.M. Budel).

https://doi.org/10.1016/j.bjp.2017.11.004

Materials and methods

Plant material

Fresh samples of aerial vegetative parts of *Piper caldense* C. DC., Piperaceae, were collected from plants growing in open and sunny areas in Paranaguá, Paraná, Brazil (54 m, 25°38′38″ S and 48°36′17″ W) in July 2014. The voucher specimen (# MBM 385665) was identified by a taxonomist [Juarez Cordeiro] and was deposited in the herbarium of Museu Botânico Municipal de Curitiba, Paraná, Brazil.

Microscopic procedure

Freshly collected leaves and stems of *P. caldense* were fixed in formalin-acetic acid-alcohol (FAA) solution (Johansen, 1940) for five days and then stored in 70% ethanol (v/v) (Berlyn and Miksche, 1976). Transverse and longitudinal sections of the samples were prepared freehand using razor blades. The sections were placed on glass slides, hydrated and double-stained with basic fuchsin and Astra blue stains (Roeser, 1962).

For histological analysis, the following standard solutions were tested: phloroglucinol/HCl for lignin (Sass, 1951), Sudan III for lipophilic compounds (Foster, 1949), and 1% iodine solution for starch (Berlyn and Miksche, 1976). Photomicrographs were prepared using digital camera (C7070) attached to a light microscope (Olympus CX 31).

For field emission scanning electron microscopy (FESEM), the samples fixed in FAA were dehydrated using increasing concentrations of ethanol. These samples were then critical point dried (Balzers CPD-030) using liquid CO_2. The completely dried samples were mounted on aluminum stubs using glued carbon tapes and then coated with gold using a Quorum SC7620 sputter coater. Photomicrographs were generated and analyzed using a Mira 3 Tescan FESEM.

An X-ray energy dispersive system (EDS), attached to the FESEM, was used for elemental analysis of calcium oxalate crystals present in the preparations of stems and leaves. This elemental microanalysis was randomly performed for the crystals as well as cells devoid of crystals (control). The morphotypes of the crystals were determined based on the work by Silva et al. (2014).

Results and discussion

As per the results obtained in this study, *P. caldense* (Fig. 1A), from the surface view of the leaf, shows straight and thin anticlinal walls on both adaxial and abaxial epidermises (Fig. 1B–E). The leaves are hypostomatic as also reported for several other *Piper* species (Gogosz et al., 2012; Raman et al., 2012; Machado et al., 2015; Santos et al., 2015; Bertocco et al., 2017; Silva et al., 2017). However, amphi-hypostomatic leaves were met in *P. sarmentosum* Roxb. (Raman et al., 2012) and amphistomatic in *P. hispidinervum* C. DC. (Gogosz et al., 2012).

Two types of stomata, namely anomocytic and tetracytic, were present with the latter type being more abundant (Fig. 1B). Tetracytic stomata were reported to be common in *Piper* species (Santos et al., 2015; Machado et al., 2015; Bertocco et al., 2017; Silva et al., 2017). However, several other types of stomata were also reported from the species of *Piper*. They are staurocytic (Souza et al., 2009), amphicyclic, anisocytic, anomocytic, polycytic, paracytic (Raman et al., 2012), cyclocytic (Silva et al., 2017) and actinocytic types (Bertocco et al., 2017). Due to the presence of multiple types of stomata, this feature may not be helpful in the delimitation of species.

During the present study, crystal sand showing bipyramidal shapes were observed externally on the adaxial leaf surface (Fig. 1F and G). This feature was not previously reported for *Piper* species. Horner et al. (2012) studied the leaves of 63 species of *Piper* and recognized crystal sand, styloids, raphides and druses. But, they have reported that these crystal types were typically confined to the palisade and spongy parenchyma tissues of the mesophyll.

In transverse section of the leaf, the epidermis is uniseriate (Fig. 2A) and is covered by a thin and smooth cuticle. A 2-layered hypodermis is found on both sides (Fig. 2A and B). In *Piper*, the number of hypodermis layers is variable. The hypodermis is uni-layered on both sides in *P. amalago* L. (Santos et al., 2015), *P. callosum* Ruiz & Pav. (Silva et al., 2017), *P. lepturum* C. DC. (Machado et al., 2015), and *P. solmsianum* C. DC. (Bertocco et al., 2017); 1–2 layered on the adaxial side in *P. sarmentosum* (Raman et al., 2012); 1–3 layered on the adaxial side in *P. betle* L. (Raman et al., 2012); 1–3 layered on the adaxial and 1–2 layered on the abaxial side in *P. aduncum* L. (Nakamura et al., 2015); 3–4 layered on the abaxial side in *P. hispidum* Sw. (Albiero et al., 2006); and 2–4 layered on both sides of the leaf in *P. arboreum* Aubl. (Souza et al., 2009).

Nakamura et al. (2015) observed that the origin of the subepidermal layers in *Piper* leaves comes from the ground meristem and, consequently, should be considered a hypodermis. They suggested that the hypodermis would have the function of regulating the intensity of light reaching the chlorenchyma. The number of hypodermal layers is considered by many authors as a taxonomic feature to identify *Piper* species (Gogosz et al., 2012; Raman et al., 2012; Santos et al., 2015; Machado et al., 2015; Bertocco et al., 2017).

Piper caldense shows pearl glands present on both the adaxial and abaxial leaf surfaces. These trichomes consist of a short stalk and an ovoid body cell measuring up to 40 μm long and 15 μm in diameter (Fig. 1D). These glandular trichomes were also found in *P. mikanianum* (Kunth) Steud (Duarte and Siebenrock, 2010), *P. betle* (Raman et al., 2012), *P. lepturum* (Machado et al., 2015), *P. amalago* (Santos et al., 2015), *P. solmsianum* (Bertocco et al., 2017), and *P. callosum* (Silva et al., 2017).

Non-glandular trichomes, multicellular and uniseriate, have been reported in many *Piper* species (Duarte and Siebenrock, 2010; Gogosz et al., 2012; Raman et al., 2014; Santos et al., 2015; Machado et al., 2015). However, this type of trichome was not observed in the present study. Considering the significant variations in the morphology of trichomes in *Piper* species, especially by the presence or absence of trichomes on the stems and leaves, as well as in terms of their shapes and sizes, this feature can be helpful in species identification in *Piper* (Monteiro and Guimarães, 2009; Gogosz et al., 2012).

The leaf is dorsiventral and is formed by two layers of palisade and 5–6 layers of spongy parenchyma (Fig. 2A and B). This feature is common in *Piper*, however, the number of layers of palisade and spongy parenchyma may vary in different species (Gogosz et al., 2012; Raman et al., 2012; Santos et al., 2015; Silva et al., 2017). The veinlets traversing the mesophyll region are represented by small collateral vascular bundles surrounded by endodermis. Secretory idioblasts with yellow lipophilic content (Fig. 2A), reacted with Sudan III in the histochemical test, are occasionally found in the lamina as well as midrib regions. Dorsiventral mesophyll and secretory idioblasts were also reported for many other *Piper* species (Gogosz et al., 2012; Raman et al., 2012; Santos et al., 2015; Machado et al., 2015; Nakamura et al., 2015; Silva et al., 2016; Bertocco et al., 2017).

In transverse section, the midrib is flat-convex in outline and is slightly angular on its abaxial side (Fig. 2C and D). This characteristic was evident in *P. betle* (Raman et al., 2012) and in *P. amalago* (Santos et al., 2015). Concave–convex (Gogosz et al., 2012) and biconvex shapes of midrib have also been found in *Piper* species (Duarte and Siebenrock, 2010; Gogosz et al., 2012; Bertocco et al., 2017; Silva et al., 2017).

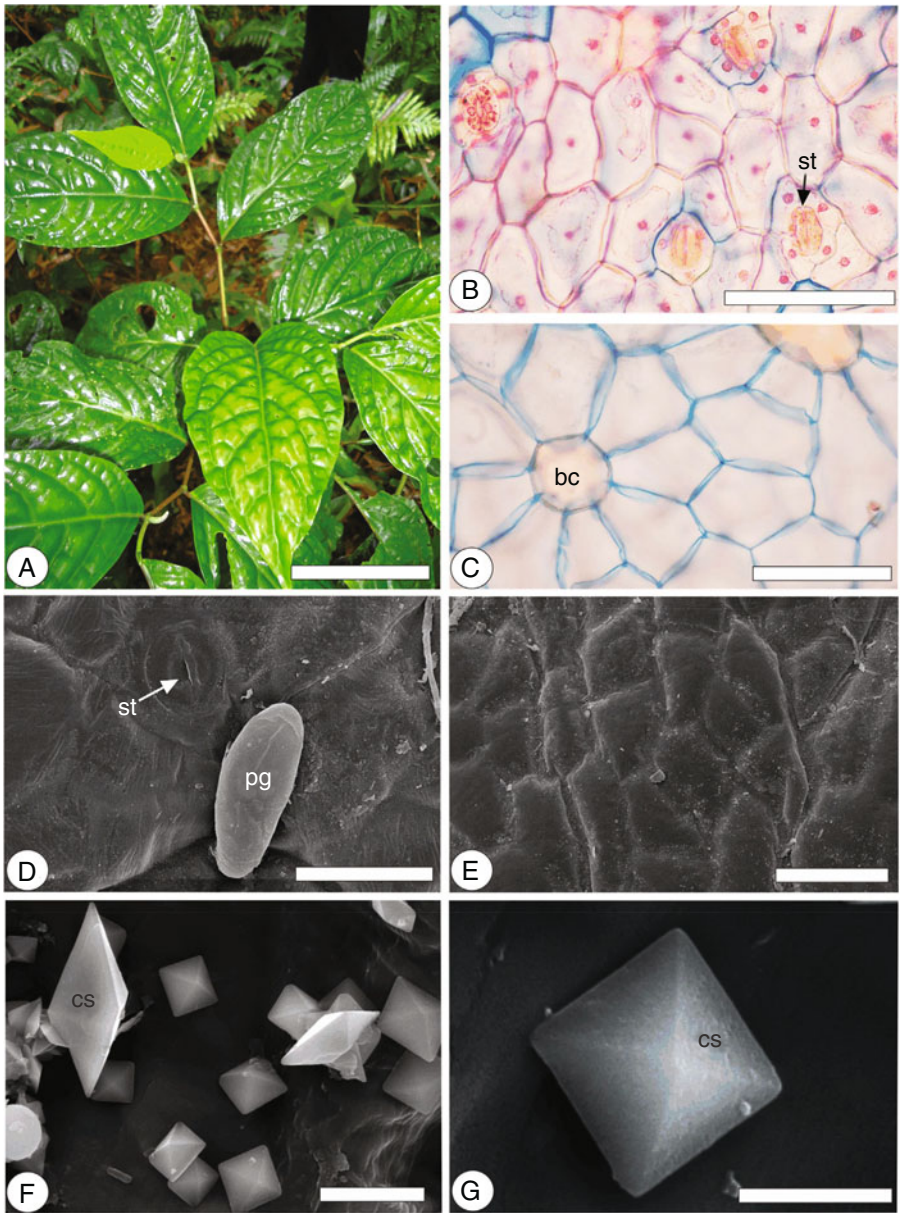

Fig. 1. Morpho-anatomy of *Piper caldense* [B, C: light microscopy; D–G: FESEM]. (A) Plant in habit. (B–G). Leaf in surface view [bc, base cell; cs, crystal sand; pg, pearl gland; st, stomata]. Scale bar: A = 5 cm; B, C, E = 50 μm; D = 25 μm; F = 5 μm; G = 2 μm.

A slightly thickened and smooth cuticle covers the single-layered epidermis. Up to 3 hypodermal layers are present in the adaxial epidermis, however, are replaced in the ridge by 1–2 patches of angular collenchyma. The palisade parenchyma cells become gradually shorter toward the middle region (Fig. 2C and D). The ground parenchyma contains secretory idioblasts (Fig. 2E) with the same characteristics previously described, and several starch grains (Fig. 2F). In the abaxial side 3–5 patches of angular collenchyma are seen (Fig. 2E).

The vascular system of the midrib is represented by up to 10 free collateral vascular bundles (Fig. 2E) arranged in U-shape in the ground parenchyma (Fig. 2C and D). Similar pattern has been described for some species, such as *P. diospyrifolium* Kunth (Souza et al., 2004), *P. lepturum* Kunth (Machado et al., 2015), *P. lindbergii* C. DC. (Gogosz et al., 2012), and *P. mikanianum* (Duarte and Siebenrock, 2010). However, the number of vascular bundles in these species is variable. *Piper callosum* showed collateral vascular bundles in a more or less straight line, centrally located in the ground parenchyma; and a solitary bundle was found in *P. sarmentosum* (Raman et al., 2012), *P. glabratum* Kunth (Gogosz et al., 2012) and *P. solmsianum* (Bertocco et al., 2017). Midrib shape and vascular pattern are useful markers to differentiate and identify *Piper* species.

The petiole, in cross-section (Fig. 3A), is slightly concave–convex in shape with two ribs on the adaxial side. The epidermis has the same features as described for the leaf blade. In the cortex, about 12 layers of angular collenchyma (Fig. 3C) are found as interrupted patches all around the petiole. The stele is represented by about 12 free collateral vascular bundles of varying sizes arranged in U-shape (Fig. 3A). Every bundle is positioned opposite to a collenchyma patch. Many secretory idioblasts are distributed in the petiole (Fig. 3C). Several starch grains that reacted positively with iodine solution (Fig. 3B), and calcium oxalate crystals are seen.

The shape of the petiole is variable in *Piper*, such as irregular in *P. amalago* (Santos et al., 2015) and heart-shaped in *P. betle* (Raman et al., 2014) and *P. callosum* (Silva et al., 2017). The vascular

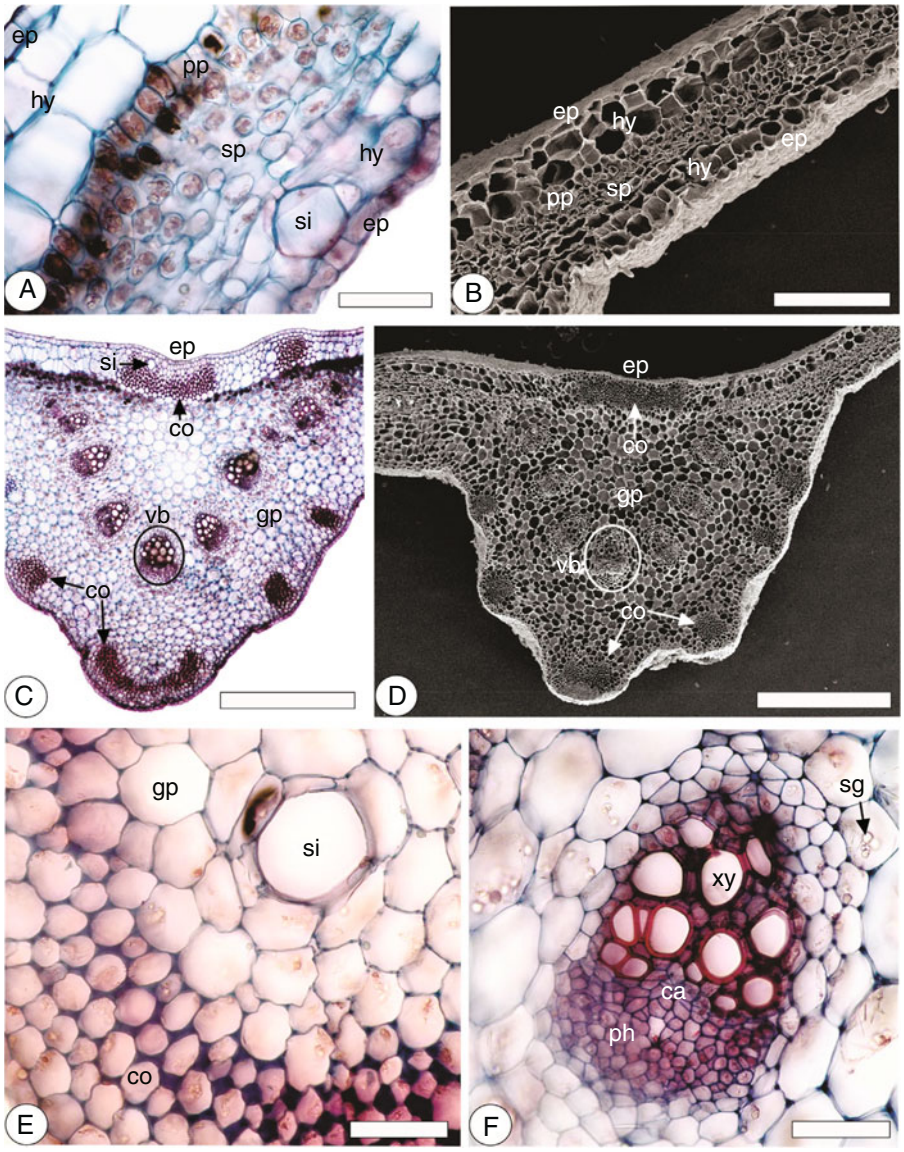

Fig. 2. Transverse sections (TS) of *Piper caldense* leaves [A, C, E–F: light microscopy; B–D: FESEM]. (A and B) TS of lamina. (C and D) TS of leaf midrib. (E and F) Enlarged views of the ground tissue (E) and a vascular bundle (F) of the midrib in TS [ca, cambia; co, collenchyma; ep, epidermis; gp, ground parenchyma; hy, hypodermis; sg, starch grains; si, secretory idioblast; ph, phloem; pp, palisade parenchyma; sp, spongy parenchyma; vb, vascular bundle; xy, xylem]. Scale bar: A, E, F = 50 μm; B, C, D = 200 μm.

pattern observed in this study was also found in other species of *Piper* (Raman et al., 2014; Machado et al., 2015; Silva et al., 2017).

In an incipient secondary structure, the stem is circular in shape (Fig. 3D). The epidermis is uniseriate and covered by a thin cuticle. Beneath the epidermis, cortical parenchyma presents 9–10 layers (Fig. 3D). In the cortex, patches of 5–8 layered angular collenchyma occur as an interrupted ring. Solitary or groups of fibers are distributed mainly in the lower parts of the collenchyma patches (Fig. 3D and E). Secretory idioblasts are also present in the cortex (Fig. 3D). The vascular system is represented by two whorls of cortical and medullary bundles. There are about 18 cortical and 12 medullary bundles separated by a continuous sclerenchymatous ring. The vascular bundles have phloem toward the periphery, and xylem facing the pith, separated by intrafascicular cambia (Fig. 3F). Perivascular fiber caps are adjoined to the phloem (Fig. 3D and F). Perivascular fiber caps, xylem and sclerenchymatous ring reacted with phloroglucinol/HCl and stained deep pink, evidencing lignification in the walls (Fig. 3D–F).

Considering Piperaceae family, Trueba et al. (2015) have affirmed that vascular bundles are arranged in two or more concentric rings in *Piper*, *Manekia* Trel., and *Zippelia* Blume. However, the number of cortical and medullary vascular bundles is variable among species. According to the pattern, *Piper* species showed this characteristic, such as about 15–23 cortical vascular bundles in *P. kadsura* (Choisy) Ohwi, 34–41 in *P. kwashoense* Hayata, around 4–7 medullary vascular bundles in *P. sintenense*, and 12–19 bundles in *P. betle* (Yang and Chen, 2017).

In *Piper* stems, the cortical vascular bundles are usually separated from the medullary bundles by a continuous sclerenchymatous ring (Duarte and Siebenrock, 2010; Santos et al., 2015; Silva et al., 2017; Yang and Chen, 2017). However, *P. betle* has showed discontinuous sclerenchymatous ring (Yang and Chen, 2017).

The pith occupies a large portion of the stem and is made up of thin-walled parenchymatous cells. Starch grains (Fig. 3E, F and I), secretory idioblasts (Fig. 3D) and crystals of calcium oxalate are found (Fig. 3G and H) in the pith region. In *Piper*, the secretory

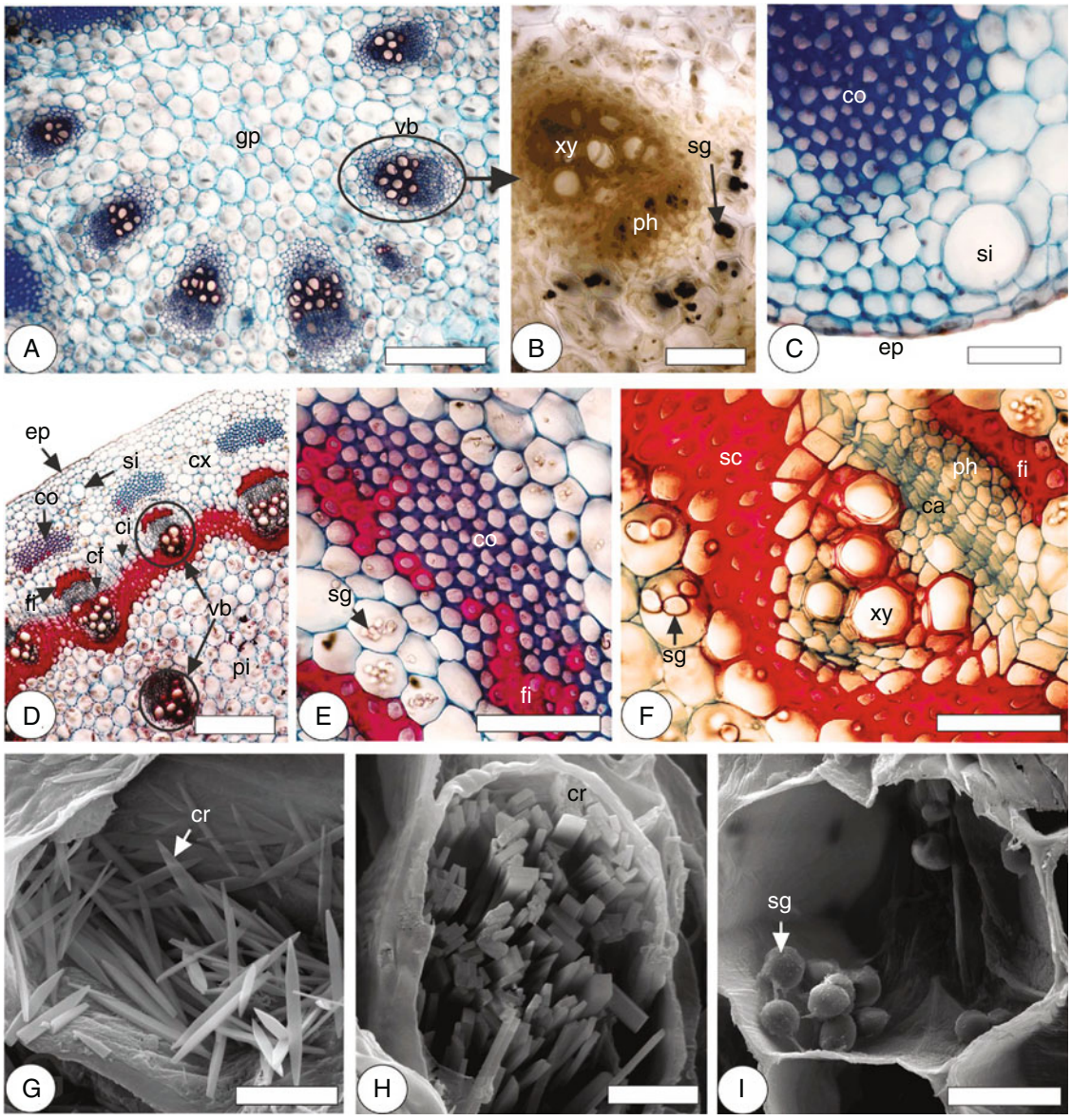

Fig. 3. Anatomy of *Piper caldense* [A–F: Light microscopy; G–I: FESEM]. (A–C) Transverse sections (TS) of petiole. (B) Starch grains in reaction with iodine solution. (D–I) TS of stem. (F) Sclerenchymatous ring and fibers in reaction with phloroglucinol/HCl [ca, cambia; cf, cambia fascicular; ci, cambia interfascicular; co, collenchyma; cr, crystal; cx, cortex; ep, epidermis; fi, fiber; gp, ground parenchyma; hy, hypodermis; ph, phloem; pi, pith; sc, sclerenchymatous ring; sg, starch grains; si, secretory idioblast; sp, spongy parenchyma; vb, vascular bundle; xy, xylem]. Scale bar: A, D = 200 μm; B, C, E, F = 50 μm; I = 20 μm; G = 5 μm; H = 2 μm.

idioblasts, crystals and starch grains are commonly found in the leaf mesophyll, ground parenchyma of the midrib and petiole as well as in the stem (Gogosz et al., 2012; Silva et al., 2014; Santos et al., 2015; Machado et al., 2015; Bertocco et al., 2017).

In the present study, *P. caldense* presents raphides (Fig. 3G and H) in the leaf midrib, petiole and stem. The raphide needles are of *Psychotria* type or Type I (Horner and Wagner, 1995; Raman et al., 2014) – four-sided and have pointed ends, measuring 7–9 μm long. In cross-section, the needles are rectangular (Fig. 3H) in shape. Raphides are common in *Piper*, but other morphotypes of crystals, such as crystal sand, cuneiform, tabular, tabular crystal rosette, styloids, styloids crystal rosette, crystal concretions, elongated square dipyramid, and very elongated square dipyramid are also found (Silva et al., 2014; Horner et al., 2015; Bertocco et al., 2017). Horner et al. (2015), combining microscopic and phylogenetic information, have identified druses and crystal sand as the two major crystals types in the order Piperales, being the other crystal types (prisms, raphides and styloids) derived from these.

The EDS spectra of the crystal sand from the leaf surface (Fig. 4A) and the raphides in the stems (Fig. 4B) of *P. caldense* show large peaks of calcium, carbon and oxygen. These results confirm that the chemical composition of these crystals is calcium oxalate as also reported by previous workers (Horner et al., 2012, 2015; Bertocco et al., 2017). However, Silva et al. (2014) have chemically analyzed the crystals from *P. arboreum* Aubl., *P. callosum*, and *P. tuberculatum* Jacq. and found pure calcium oxalate, mixtures of oxalates and sulfates, and mixtures of oxalates, sulfates and silica. The unlabeled peaks in the spectra (Fig. 4A and B) represent conductive metal used for coating the samples for SEM analysis.

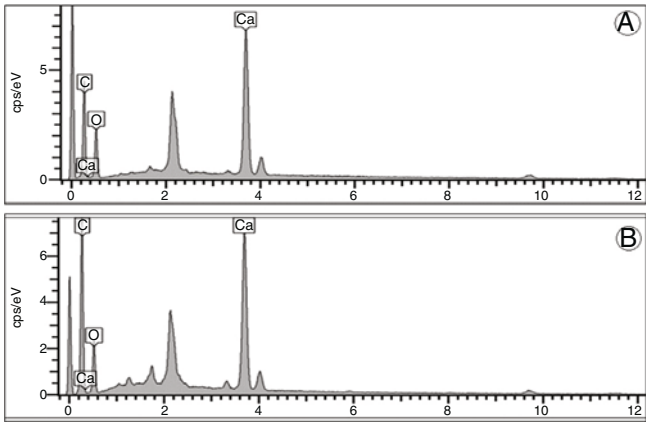

Fig. 4. X-ray energy dispersive elemental analysis of isolate crystals – (A) crystal sand and (B) raphides.

Conclusion

The present study revealed the following anatomical features that can be used for the identification of *P. caldense*. The leaves are hypostomatic and possess a 2-layered hypodermis. Unicellular pearl glands are found on the leaf surfaces. The midrib is flat-convex, with ten vascular bundles arranged in U-shape. The petiole is slightly concave–convex with two ribs on the adaxial side, and has a U-shaped stele consisting of twelve vascular bundles. The stem is circular and has a continuous ring of sclerenchymatous sheath in the pith. Calcium oxalate sand crystals are observed on the adaxial leaf surface, and raphides are found in the leaf midrib, petiole and stem.

Authors' contributions

VLPS collected and identified the plant material. VLPS and VBB carried out the laboratory work. IPM and CRCF performed SEM analysis. VR and IAK provided critical reading and insightful recommendations of the manuscript. JMB created the project, supervised the laboratory work, and wrote the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

We acknowledge the Electron Microscopy Center of the LABMU at the State University of Ponta Grossa and the Electron Microscopy Center of the Federal University of Paraná for assistance in preparing SEM images and EDS spectra.

References

Albiero, A.L.M., Paoli, A.A.S., Souza, L.A., Mourão, K.S.M., 2006. Morfoanatomia dos órgãos vegetativos de *Piper hispidum* Sw. (Piperaceae). Rev. Bras. Farmacogn. 16, 379–391.

Alves, H.S., Rocha, W.R.V., Fernandes, A.F.C., Nunes, L.E., Pinto, D.S., Costa, J.I.V., Chaves, M.C.O., Catão, R.M.R., 2016. Antimicrobial activity of products obtained from *Piper* species (Piperaceae). Rev. Cubana Plantas Med. 21, 168–180.

Araújo, M.J., Câmara, C.A., Born, F.S., Moraes, M.M., Badji, C.A., 2012. Acaricidal activity and repellency of essential oil from *Piper aduncum* and its components against *Tetranychus urticae*. Exp. Appl. Acarol. 57, 139–155.

Berlyn, G.P., Miksche, J., 1976. Botanical Microtechnique and Cytochemistry. Iowa State University, Eames.

Bertocco, A.R.P., Migacz, I.P., Santos, V.L.P., Franco, C.R.C., Silva, R.Z., Yunes, R.A., Cechinel-Filho, V., Budel, J.M., 2017. Microscopic diagnosis of the leaf and stem of *Piper solmsianum* C. DC. Microsc. Res. Tech. 80, 831–837.

Cardozo Júnior, E.L., Chaves, M.C.O., 2003. Caldensin, a new natural *N*-methylaristolactam from *Piper caldense*. J. Pharm. Biol. 41, 216–218.

Carrara, V.S., Serra, L.Z., Cardozo-Filho, L., Cunha-Júnior, E.F., Torres-Santos, E.C., Cortez, D.A.G., 2012. HPLC analysis of supercritical carbon dioxide and compressed propane extracts from *Piper amalago* L. with antileishmanial activity. Molecules 17, 15–33.

Duarte, M.R., Siebenrock, M.C.N., 2010. Caracteres anatômicos de folha e caule de *Piper mikanianum* (Kunth) Steud., Piperaceae. Lat. Am. J. Pharm. 29, 45–51.

Freitas, L.A.B., Aquino, F.S., Araújo, J.M., Ramos, C.S., 2016. Chemical profiles and antimicrobial activity of *Piper caldense* tissues. Ann. Chromatogr. Sep. Tech. 2, 1026–1028.

Foster, A.S., 1949. Practical plant anatomy, 2nd ed. D. Van Nostrand, New Jersey, Princeton.

Guimarães, E.F., Carvalho-Silva, M., Monteiro, D., Medeiros, E.S., Queiroz, G.A., 2015. Piperaceae in Lista de Espécies da Flora do Brasil. Jardim Botânico do Rio de Janeiro, http://floradobrasil.jbrj.gov.br/jabot/floradobrasil/FB12735 (accessed August 2017).

Gogosz, A.M., Boeger, M.R.T., Negrelle, R.R.B., Bergo, C., 2012. Anatomia foliar comparativa de nove espécies do gênero *Piper* (Piperaceae). Rodriguésia 63, 405–417.

Horner, H.T., Wagner, B.L., 1995. Calcium oxalate formation in higher plants. Calcium Oxalate in Biological Systems, vol. 1., pp. 53–72.

Horner, H.T., Wanke, S., Samain, M.S., 2012. A comparison of leaf crystal macropatterns in the two sister genera *Piper* and *Peperomia* (Piperaceae). Am. J. Bot. 99, 983–997.

Horner, H.T., Samain, M.S., Wagner, S.T., Wanke, S., 2015. Towards uncovering evolution of lineage-specific calcium oxalate crystal patterns in Piperales. Botany 93, 159–169.

Johansen, D.A., 1940. Plant Microtechnique. McGraw Hill Book, New York, NY.

Machado, N.S.O., Pereira, F.G., Santos, P.R.D., Costa, C.G., Guimarães, E.F., 2015. Comparative anatomy of the leaves of *Piper lepturum* (Kunth) C. DC. var. *lepturum* and *Piper lepturum* var. *angustifolium* (C.DC.) Yunck. Hoehnea 42, 1–8.

Marques, A.M., Barreto, A.L.S., Curvelo, J.A.R., Velozo, L.S.M., Moreira, D.L., Guimarães, E.F., Soares, R.M.A., Kaplan, M.A.C., 2010. Biological activity and chemistry of essential oils from *Piper claussenianum* essential oil (Piperaceae). Nat. Prod. Commun. 11, 1837–1840.

Marques, A.M., Kaplan, M.A.C., 2015. Active metabolites of the genus *Piper* against *Aedes aegypti*: natural alternative sources for dengue vector control. Univ. Sci. 20, 61–82.

Monteiro, D., Guimarães, E.F., 2009. Flora do Parque Nacional do Itatiaia – Brasil: *Manekia* e *Piper* (Piperaceae). Rodriguésia 60, 999–1024.

Nakamura, A.T., Simão, E., Silva, L., Torres, G.A., 2015. Origin of the subepidermal tissue in *Piper* L. leaves. Braz. J. Biol. 75, 368–371.

Pereira, R., Guedes, A., Da Silva, G.E., 2015. The hydroalcoholic extract of leaves of *Piper caldense* C. DC. decreases alcohol consumption in rats. Rev. Bras. Plantas Med. 17, 157–163.

Raman, V., Galal, A.M., Khan, I.A., 2012. An investigation of the vegetative anatomy of *Piper sarmentosum*, and a comparison with the anatomy of *Piper betle* (Piperaceae). Am. J. Plant Sci. 3, 1135–1144.

Raman, V., Horner, H.T., Khan, I.A., 2014. New and unusual forms of calcium oxalate raphide crystals in the plant kingdom. J. Plant Res. 127, 721–730.

Rocha, D.S., Silva, J.M., Navarro, D.M.A.F., Camara, C.A.G., Lira, C.S.L., Ramos, C.S., 2016. Potential antimicrobial and chemical composition of essential oils from *Piper caldense* tissues. J. Mex. Chem. Soc. 60, 148–151.

Roeser, K.R., 1962. Die Nadel der Schwarzkiefer-Massenprodukt und Kunstwerk der Natur. Mikrokosmos 61, 33–36.

Santos, V.L.P., Franco, C.R.C., Amano, E., Messias-Reason, I.J., Budel, J.M., 2015. Anatomical investigations of *Piper amalago* (jaborandi-manso) for the quality control. Rev. Bras. Farmacogn. 25, 85–91.

Santos, V.L.P., Lima, C.P., Campos, R., Ribeiro, C.S.R., Marques, F.A., Budel, J.M., Messias-Reason, I.J., 2016. Chemical composition and antimicrobial activity of volatile oils of *Piper amalago* L. Lat. Am. J. Pharm. 35, 1883–1889.

Sarnaglia-Junior, V.B., Bermudez, G.M.M., Guimarães, E.F., 2014. Diversidade de Piperaceae em um remanescente de Floresta Atlântica na região serrana do Espírito Santo, Brasil. Biotemas 27, 49–57.

Silva, R.J.F., Aguiar-Dias, A.C.A., Mendonça, M.S., 2014. Rosetas e concrescências cristalinas silicificadas em *Piper* (Piperaceae): registros inéditos de macropadrões. Acta Amaz. 44, 435–446.

Silva, R.J.F., Aguiar-Dias, A.C.A., Faial, K.C.F., Mendonça, M.S., 2016. Caracterização farmacognóstica de *Piper arboreum* var. *arboreum* e *P. tuberculatum* (Piperaceae). Acta Amaz. 46, 195–220.

Silva, R.J.F., Aguiar-Dias, A.C.A., Faial, K.C.F., Mendonça, M.S., 2017. Morphoanatomical and physicochemical profile of *Piper callosum*: valuable assessment for its quality control. Rev. Bras. Farmacogn. 27, 20–33.

Souza, L.A., Moscheta, I.S., Oliveira, J.H.G., 2004. Comparative morphology and anatomy of the leaf and stem of *Peperomia dahlstedtii*, *Ottonia martiana* and *Piper diospyrifolium* (Piperaceae). Gayana Bot. 6, 6–17.

Souza, L.A., Albiero, A.L.M., Almeida, O.J.G., Lopes, W.A.L., Mourão, K.S.M., Moscheta, I.S., 2009. Estudo morfo-anatômico da folha e do

caule de *Piper arboreum* Aubl. (Piperaceae). Lat. Am. J. Pharm. 28, 103–107.
Takahashi, F.Y., Ohlweiler, F.P., Kawano, T., 2013. Efeitos de extratos de Piperaceae em *Biomphalaria glabrata* (Say, 1818) (*Mollusca: Planorbidae*). BEPA 10, 31–32.
Trueba, S., Rowe, N.P., Neinhuis, C., Wanke, S., Wagner, S.T., Isnard, S., 2015. Stem anatomy and the evolution of woodiness in Piperales. J. Plant Sci. 176, 468–485.
Yang, S.Z., Chen, P.H., 2017. Cambial variations of *Piper* (Piperaceae) in Taiwan. Bot. Stud. Int. J. 58, 1–9.