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+ Corresponding author: italo.coutinho@ufc.br

A safer and more sustainable methodology for herbarium specimen rehydration in morphoanatomical studies

Ítalo Antônio Cotta Coutinho [1], Marcus Levy Sanders Fernandes [1], Lara Luiza Sales Alves [1], Antonia Mirelle Lopes Marques [1], Valdnéa Casagrande Dalvi [2]

[1] Universidade Federal do Ceará
[2] Instituto Federal de Educação, Ciência e Tecnologia Goiano

Abstract. Morphoanatomical studies based on herbarium material depend on rehydration procedures, in which the use of 2% potassium hydroxide (KOH) after boiling is common practice. Although this reagent facilitates tissue rehydration, it is a strong and corrosive base, capable of damaging sensitive tissues besides being associated with environmental concerns. Therefore, the adoption of methodologies that reduce or even eliminate its use is desirable. In this study, we evaluated the suppression of KOH during the rehydration of herbarium specimens. Leaf samples of different types (i.e. papery, coriaceous and succulent leaves, either glabrous or pubescent) were dried and subsequently rehydrated without alkaline treatment. Freehand sections were clarified with 50% sodium hypochlorite, stained with Astra blue and safranin, mounted on temporary slides, and examined under light microscopy. The material exhibited structural integrity, allowing detailed visualization of leaf anatomical features. The elimination of KOH proved to be effective, resulting in a simpler, faster, and more environmentally friendly procedure.
Keywords: anatomical techniques, leaf, light microscopy, plant anatomy, sustainability

Introduction

Scientific collections comprise sets of organisms or parts thereof that are properly preserved and catalogued for didactic and scientific purposes (SiBBR, 2026). Botanical collections, for example, document research across different fields of knowledge, such as taxonomic and floristic studies, and function as important databases capable of storing representative information on species and even on natural and/or cultivated populations (Gadelha Neto *et al.*, 2013; Monteiro; Siani, 2009). In this context, herbaria constitute biological collections that are organized and preserved according to specific classification systems, serving as repositories for the deposition and maintenance of herbarium specimens (Fidalgo; Bononi, 1984; Gadelha Neto *et al.*, 2013). Thus, herbarium materials play a fundamental role in several areas of botany and related fields (Dias *et al.*, 2019).

For a plant specimen to be incorporated into a herbarium collection, the first step consists in drying the fresh material, that is, placing the specimen in a plant press for subsequent dehydration and preparation for deposition (Gadelha Neto *et al.*, 2013; IBGE, 2012). During the dehydration process used to produce voucher material, samples are dried in forced-air oven (or even in a simple drier constructed with light bulbs as the heat source) until complete removal of water (Gadelha Neto *et al.*, 2013; IBGE, 2012). However, when collections are carried out in remote or inhospitable areas, the lack of access to drying ovens at the time of pressing may represent a methodological constraint. In such cases, to prevent fungal growth or tissue decay, pressed specimens are often sprayed or moistened with ethanol – or even with high-alcohol-content beverages such as rum and *cachaça* – until they can be transferred to a proper drier (Hodge, 1947; Rotta; Carvalho; Zonta, 2008; Schultes, 1947). After dehydration, the herbarium material is properly mounted on a sheet of paper and is then referred to as an exsiccate, which receives a specific accession number, is

catalogued, and incorporated into the herbarium collection (IBGE, 2012).

Herbarium specimens often represent the only available source of material for plant anatomical studies (Meira; Martins, 2003), particularly in investigations that use anatomy as a complementary tool to classical taxonomy based on morphological characters (Coutinho *et al.*, 2016; Dalvi *et al.*, 2014; Dalvi; Meira; Azevedo, 2013; Pinto-Silva *et al.*, 2023; Vitarelli *et al.*, 2015). Such studies frequently involve many species and replicates, including rare or endemic taxa, or material originating from distant localities, such as other states, countries, or continents (Gonçalves *et al.*, 2024; Inceer; Ozcan, 2021; Kolb; Gomes; Lombardi, 2020; Mouzella *et al.*, 2025), making the collection of fresh material impractical. In this context, herbaria play a central role as facilitators of this type of research.

The use of herbarium material in anatomical studies requires the reversal of the herbarium preservation process (Meira; Martins, 2003; Smith; Smith, 1942), allowing specimens to be sectioned according to standard plant anatomical methodologies. This reversal begins with the rehydration of the material, which is boiled in distilled water and subsequently treated overnight with 2% potassium hydroxide (KOH), promoting complete tissue expansion (Meira; Martins, 2003; Smith; Smith, 1942). However, the use of KOH on fragile materials, such as rehydrated herbarium specimens, may be problematic. As a strong and highly corrosive base (Atkins; Jones, 2008), KOH can compromise tissue integrity. Observations made over several years (unpublished data) indicate that materials subjected to KOH treatment, even for periods shorter than overnight, become excessively fragile and softened, hindering subsequent handling and, in some cases, leading to sample disintegration.

In addition to the potential damage to plant material, potassium hydroxide (KOH) is typically produced in the chlor-alkali industry through the electrolysis of brine (KCl) (Garcia-Herrero *et al.*, 2017). The production of KOH is associated with several drawbacks, including high electricity consumption (Boulamanti; Moya, 2017; Garcia-Herrero *et al.*, 2017) and the release of chlorine gas (Cl_2) as a highly toxic coproduct (Rostrup-Nielsen; Sehested; Noerskov, 2003). Potassium hydroxide is also toxic to humans (Korsaga-Somé *et al.*, 2015) and may cause environmental impacts when improperly discarded, such as increasing soil pH (Agourakis *et al.*, 2006).

In this context, the development of methodologies that minimize or eliminate the use of this reagent becomes desirable. Accordingly, the present study aimed to reduce the negative effects of the rehydration process commonly used in morphoanatomical studies by omitting KOH. In doing so, we refine herbarium specimen rehydration and propose an effective, safer, and more environmentally sustainable methodology.

Material and Methods

Sample collection and preparation

To cover a broader range of leaf types, six model species were selected, representing six distinct leaf categories: (1) papery and glabrous, *Citrus* × *limon* (L.) Osbeck (Figure 1A); (2) papery and pubescent, *Cucurbita pepo* L. (Figure 1B); (3) coriaceous and glabrous, *Clusia fluminensis* Planch. & Triana (Figure 1C); (4) coriaceous and pubescent, *Adenium obesum* (Forssk.) Roem. & Schult. (Figure 1D); (5) succulent and glabrous, *Kalanchoe brasiliensis* Cambess. (Figure 1E); and (6) succulent and pubescent, *Plectranthus* sp. L'Hér. (Figure 1F). Leaf textures were classified according to Souza et al. (2019). For each leaf category, at least three leaves were used per treatment, providing three replicates.

Considering that samples intended for morphoanatomical studies, when derived from herbarium material, may have been subjected to two distinct drying procedures (i.e. with or without prior ethanol treatment), both procedures were adopted in this study. Half of the samples of each leaf type were subjected to two drying methods: (1) samples were pressed and immediately placed in the drier (45–48 °C) using light bulbs as the source of heat until complete dehydration; and (2) samples were pressed, placed in plastic bags, and moistened with commercial ethyl alcohol (92° GL) for seven days, after which they were placed in the drier until complete dehydration. In both procedures, the drier operated only during the daytime period, between 08:00 and 17:00.

Rehydration and anatomical analysis

After drying, the samples were placed in beakers containing boiling distilled water and kept under boiling conditions until they became fully submerged or for a maximum of seven minutes, in order to avoid tissue cooking. Subsequently, the samples remained in the water until complete cooling (Smith; Smith, 1942). Rehydrated leaves derived from both drying procedures (i.e. with or without ethanol treatment) were then divided into two groups. Half of the samples remained in distilled water, whereas the other half was subjected to a 2% potassium hydroxide (KOH) solution for eight hours and subsequently rinsed three times in distilled water, for 10 minutes each rinse. Both the material maintained in distilled water and that treated with KOH were dehydrated through a graded ethanol series (10%, 30%, 50%, and 70% ethyl alcohol), remaining for 30 minutes at each concentration. After this procedure, the samples were stored in 70% ethanol until sectioning.

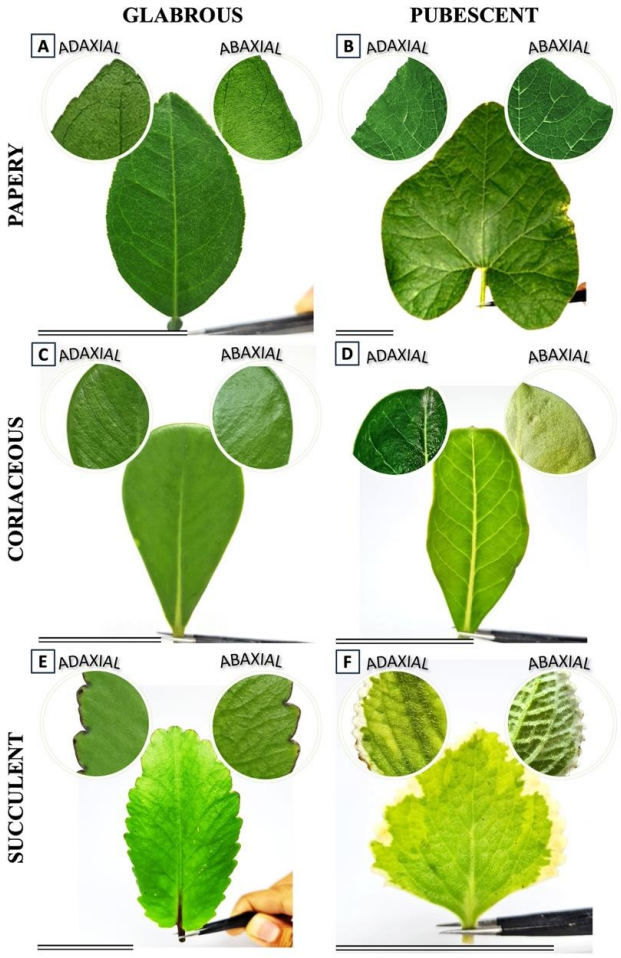

Figure 1. Leaf types represented by the model species used in this study. A: *Citrus* × *limon*; B: *Cucurbita pepo*; C: *Clusia fluminensis*; D: *Adenium obesum*; E: F: *Kalanchoe brasiliensis*; *Plectranthus* sp. Asterisks (*) indicate specimens that were subjected to alcohol previously to drying. Scale bars: 5 cm

Freehand sections were obtained from the midrib region using disposable razor blades, with the petiole of *Cecropia palmata* Willd. used as a support (Silva *et al.*, 2013). This support is as delicate as the stem pith of *Tetrapanax papyrifer* (Hooker) K. Koch used in many countries (Sousa *et al.*, 2025). The sections were cleared in a sodium hypochlorite solution (50% v/v, prepared by dilution of household bleach) and rinsed in distilled water until complete removal of the characteristic odor of the reagent, with a minimum of four washes of 5 minutes each (Kraus; Ardiun, 1997). Subsequently, the samples were stained with safrablau (a mixture of 1% safranin and 2% Astra blue) for one minute (Kraus; Ardiun, 1997), quickly rinsed in distilled water to remove excess stain, and mounted between slide and coverslip with distilled water for observation under a light microscope.

The sections were examined under a light microscope (PHYSIS UB103i, China) equipped with an attached digital camera for image acquisition (OIYU MC-5100, China). The entire methodology adopted is summarized in a flowchart (FIGURE 2).

Results and discussion

Methodology employed

The drying time of the samples in the drier ranged from a few hours to seven days. Samples subjected to prior alcohol treatment showed shorter dehydration times in the drier, not exceeding two days, whereas those that did not receive this treatment required up to seven days of drying.

Regarding the rehydration process, no differences in the immersion time were observed among the samples, regardless of whether the dried specimens had been previously subjected to alcohol treatment or not. In general, irrespective of the methodology employed, the materials sectioned after rehydration remained structurally intact, except for the pubescent coriaceous leaf sample pressed with 92° GL alcohol and subsequently subjected to KOH treatment. The anatomical sections obtained allowed the visualization of all leaf tissues, both in the midrib region and in the remaining portions of the lamina.

Samples treated with alcohol, whether or not subsequently subjected to KOH after rehydration, showed greater fragility during sectioning, being more friable. However, complete sections were obtained for all materials, except for the pubescent coriaceous leaf sample pressed with 92° GL alcohol and subjected to KOH after

rehydration.

In the drying methodology followed exclusively by rehydration in water, sectioning was easier for all leaf types when compared to the other methodologies, as the rehydrated leaves exhibited greater consistency. Materials subjected to KOH treatment appeared softer during sectioning; in this case, the only material for which intact sections could not be obtained corresponded to the pubescent coriaceous leaf previously treated with KOH.

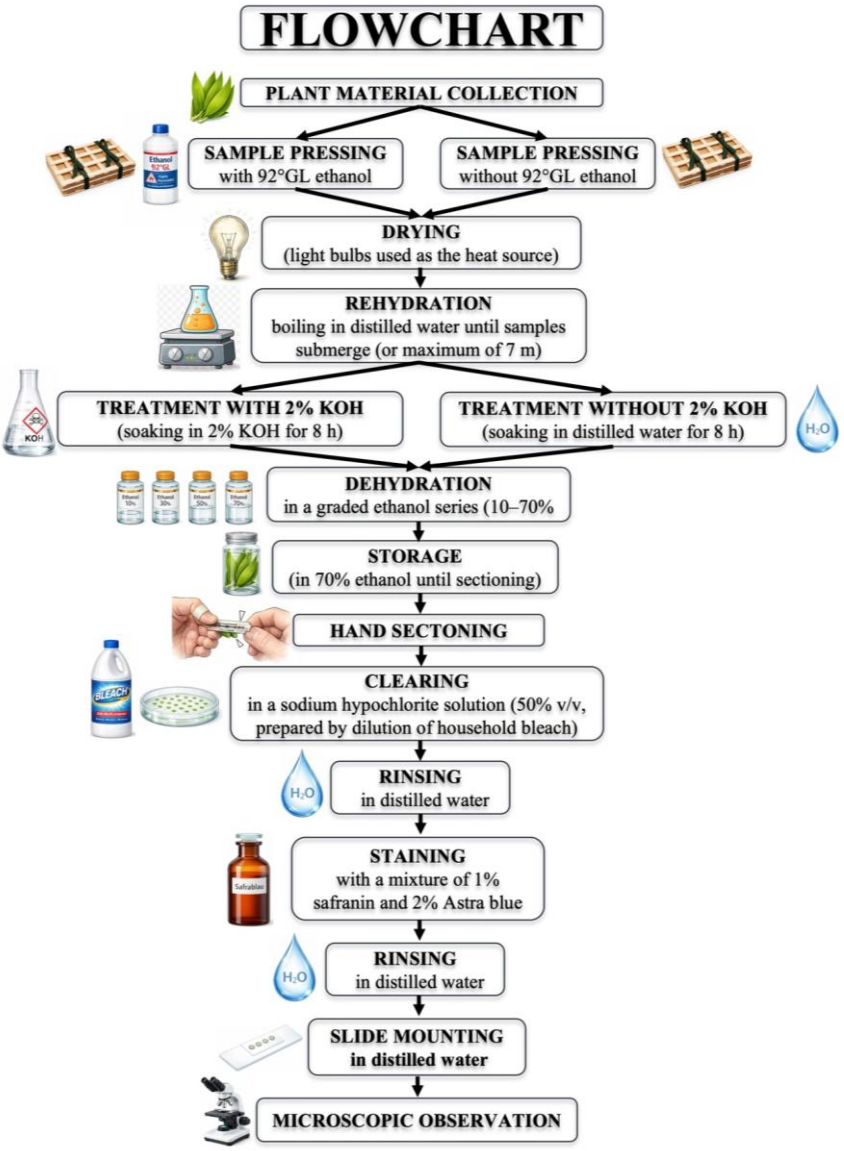

Figure 2. Flowchart summarizing the experimental workflow used for rehydration, chemical treatment, and anatomical preparation of plant material for hand-sectioning and light microscopy.

Leaf samples bearing trichomes were more difficult to section than glabrous leaves, regardless of the techniques employed (pressing with or without alcohol and application or omission of KOH). Glabrous leaves yielded more intact sections, whereas partial detachment of the epidermis was observed in some sections of pubescent leaves.

Anatomical characterization of the model species

In cross sections of the leaf of *Citrus* × *limon* (papery, glabrous) (FIGURE 3A, B), a typical dorsiventral leaf structure was observed. The epidermis of both the adaxial and abaxial surfaces was uniseriate, with stomata restricted to the abaxial surface. Idioblasts were observed beneath both the adaxial and abaxial sides of the epidermis, some containing calcium oxalate crystals; those located beneath the abaxial side of the epidermis were smaller than those facing the adaxial surface (Figure 3B). The mesophyll was dorsiventral, with palisade parenchyma composed of two, rarely three, cell layers and spongy parenchyma consisting of eight to ten layers (Figure 3B). Secretory cavities (Figure 3A, B) were observed predominantly toward the adaxial surface, although they also occurred on the abaxial surface. In the midrib region (FIGURE 3A),

the vascular system exhibited an organization similar to a eustele, with a central parenchymatic pith surrounded by xylem and externally by phloem; this eustelic structure was delimited by fibers.

The leaf of *Cucurbita pepo* (papery, pubescent), when observed in cross section (Figure 3C–E), exhibited a uniseriate epidermis bearing both non-glandular and glandular trichomes (Figure 3C–E); glandular trichomes were less abundant and more sparsely distributed. Stomata were present on both leaf surfaces, characterizing the leaf as amphistomatic. In the midrib region (Figure 3C), layers of angular collenchyma were observed beneath the epidermis on both surfaces. The mesophyll was dorsiventral and, although the thicknesses of the palisade and spongy parenchyma were similar, the palisade parenchyma consisted of two cell layers, whereas the spongy parenchyma comprised three to five layers (Figure 3E). In the midrib (Figure 3C), the vascular bundles were bicollateral.

Leaf cross sections of *Clusia fluminensis* (coriaceous, glabrous) (Figure 3F–H), exhibited a uniseriate epidermis, with stomata restricted to the abaxial surface, characterizing the leaf as hypostomatic. On the adaxial surface, three to six hypodermal layers were observed, most commonly five; these layers lacked chloroplasts and displayed features like water-storage parenchyma (FIGURE 3F, G). The mesophyll was dorsiventral, generally composed of eight layers of palisade parenchyma and approximately fifteen layers of spongy parenchyma (FIGURE 3F). Small secretory ducts were observed in both the mesophyll and the midrib (FIGURE 3F, H). Numerous druses were distributed throughout the mesophyll but were absent from the water-storage parenchyma layers located immediately beneath the epidermis on the adaxial surface. The vascular system consisted of collateral bundles surrounded by fibers (FIGURE 3H).

Adenium obesum (coriaceous, pubescent) (Figure 3I, J), leaf cross sections showed uniseriate epidermis composed of large, rectangular epidermal cells elongated in the vertical direction and highly vacuolated (Figure 3I, J). The epidermis was covered by a relatively thin cuticle, with thickening of the outer periclinal cell wall on the adaxial surface, corresponding to approximately one fifth of the height of the epidermal cells (Figure 3I, J). Stomata were observed only on the abaxial surface, characterizing the leaf as hypostomatic. The mesophyll showed a dorsiventral organization (Figure 3I), with two, rarely three, layers of palisade parenchyma on the adaxial surface and between ten and fourteen layers of spongy parenchyma on the abaxial surface. Elongated unicellular trichomes were observed on the abaxial surface, (Figure 3I) reaching approximately half of the mesophyll thickness. In the midrib region, the vascular system consisted of bicollateral vascular bundles with a concave configuration (Figure 3J), associated with one to five layers of collenchyma, more numerous on the abaxial surface.

In *Kalanchoe brasiliensis* (succulent, glabrous), the leaf observed in cross section (Figure 3K, L) exhibited a uniseriate epidermis, with stomata restricted to the abaxial surface. The mesophyll was homogeneous (Figure 3I, J), composed of numerous cell layers, with a minimum of thirteen layers formed by large cells and with reduced or absent intercellular spaces. The vascular bundles were collateral (Figure 3I, J). In the midrib region, vascular bundles of larger caliber were associated with layers of angular collenchyma adjacent to the epidermis.

Leaf cross sections of *Plectranthus* sp. (succulent, pubescent) (Figure 3M–P) showed a uniseriate epidermis on both leaf surfaces, composed of small epidermal cells with a markedly sinuous contour on the abaxial surface (FIGURE 3M, O). Both non-glandular and glandular trichomes were observed on both surfaces (Figure 3M–P). Although the leaf was characterized as amphistomatic, stomata occurred predominantly on the abaxial surface. The mesophyll exhibited a homogeneous organization (Figure 3M, O), with a predominance of three to four layers of more columnar cells on the adaxial surface, without the formation of a typical palisade, and seven to eight layers of more polyhedral cells on the abaxial surface. The vascular bundles were collateral, both in the midrib and in the median region of the mesophyll. In the midrib region, two, rarely three, layers of collenchyma were observed.

Overall, we demonstrated that omitting KOH from the rehydration process of herbarium material for anatomical studies is adequate, as it allowed the obtention of intact sections, including those produced by freehand sectioning. This condition enabled reliable anatomical descriptions for all leaf types analyzed, indicating that the adopted method preserves the structural integrity required for detailed anatomical characterization.

The anatomical descriptions of the studied species were largely consistent with those reported in the literature, either for the species themselves or, when specific studies were unavailable, for their respective genera, including *Citrus* × *limon* (Storey; Leigh, 2004), *Cucurbita pepo* (Silva, 2023), *Clusia fluminensis* , *Adenium obesum* (Carvalho, 2022; El-Taher *et al.*, 2020; Omino, 1996), *Kalanchoe brasiliensis* (Moreira *et al.*, 2012) and *Plectranthus* sp.(Duarte; Lopes., 2007; Mauro *et al.*, 2008). These comparisons reinforce our results, indicating that it was possible to describe the anatomy of all studied species using the proposed methodology.

Our results indicate that KOH treatment after rehydration can be associated with a reduction in the quality of anatomical sections, with effects that were species dependent. This was particularly evident in *Adenium obesum* (coriaceous, pubescent), for which intact leaf sections could not be obtained when the material was treated with KOH, regardless of whether it had been previously subjected to ethyl alcohol during the oven-drying process. As a strong base, KOH may promote

chemical alterations in cell wall components, especially cellulose, due to its corrosive nature (Atkins; Jones, 2008; Dehaut *et al.*, 2016), which may explain the increased tissue fragility observed in this species.

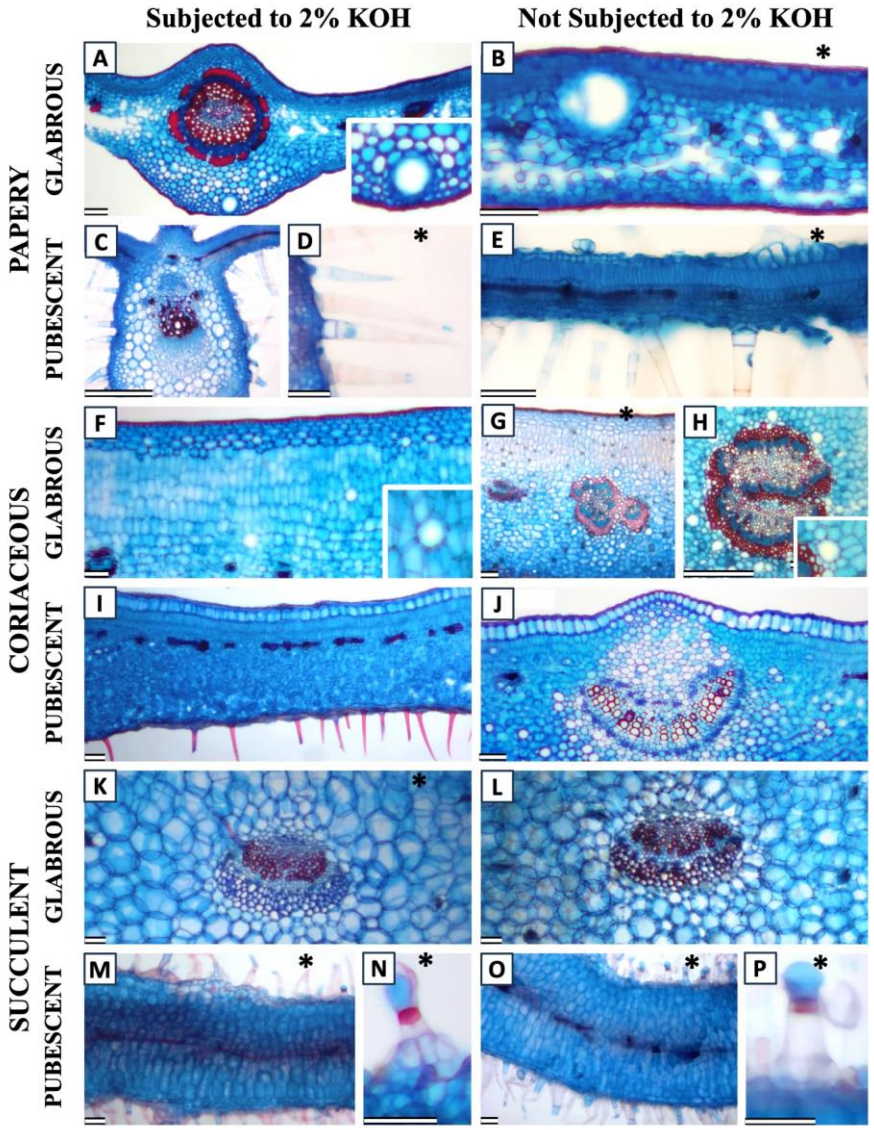

Figure 3. Leaf cross sections of all leaf types represented by the model species used in this study. A, B: *Citrus* × *limon*, detail a secretory cavity in A; C–E: *Cucurbita pepo*; F–H: *Clusia fluminensis*, detail secretory ducts in F and H; I, J: *Adenium obesum*. K, L: *Kalanchoe brasiliensis*; M–P: *Plectranthus* sp., detail of secretory trichomes in O and P. Scale bars = 100 µm.

Although KOH, even at concentrations as high as 10%, cannot readily dissolve plant materials (Herrera et al. 2018) – a concentration higher than that used in the present study – its use, even at low concentrations, may be detrimental to tissue preservation. Therefore, the differences observed in relation to the results reported by Smith and Smith (1942) indicate that this protocol is not universally applicable.

It is therefore desirable that KOH be replaced by other substances or by alternative laboratory methodologies, since, as discussed above, this compound is a potential source of environmental damage as well as being associated with high production costs (Agourakis *et al.*, 2006; Boulamanti; Moya, 2017; Garcia-Herrero *et al.*, 2017; Rostrup-Nielsen; Sehested; Noerskov, 2003). In humans, contact of this substance with the skin and mucous membranes can cause irritation, burns, and even tissue necrosis (Korsaga-Somé *et al.*, 2015), indicating that its handling in the laboratory requires caution.

Especially in studies of anatomy applied to taxonomy, in which the number of species derived from herbarium material is generally high, a considerable amount of KOH is used during the rehydration of herbarium material. Moreover, KOH is not always disposed of properly, which may contribute to increases in soil pH (Agourakis *et al.*, 2006) or in aquatic systems.

Although leaves vary in size and, consequently, the volume of 2% KOH required for the rehydration process also varies, illustrative calculations can be made. As an example, a study involving the genus *Chamaecrista* Moench used 21 herbarium specimens (Coutinho et al. 2013). If approximately 100 mL of 2% KOH were used for each specimen, this would result in a total of 2.1 L of 2% KOH. For proper disposal, neutralization with an acid is required, with hydrochloric acid (HCl) being the most used. However, because HCl is a strong acid, a simpler, cheaper, less toxic, and effective alternative is the use of vinegar ($KOH + CH_3COOH \rightarrow CH_3COOK + H_2O$). To neutralize 2.1 L of 2% KOH, approximately 1 L of culinary vinegar would be required, in which the concentration of acetic acid is typically 4–6%. This procedure would therefore generate two waste streams: the KOH solution used during rehydration and the vinegar used for neutralization.

Leaves bearing trichomes represented a greater difficulty during histological hand-sectioning. This difficulty could be explained by the presence of compounds that promote cell wall hardening in trichomes, such as structural lipids. However, none of the trichome-bearing leaves examined showed thickened periclinal walls, ruling out this explanation. Thus, the difficulty in obtaining sections may instead be attributed to the increased surface contact caused by the presence of trichomes, which increases friction between the material and the razor blade at the moment of sectioning (Liang *et al.*, 2021). This effect is evident when trichome-covered leaves from fresh material are sectioned during routine work in plant anatomy laboratories.

Regardless of leaf type, pressing material that had been conditioned in ethanol prior to drying made subsequent sectioning more difficult due to the material friability. However, after the hydration, the best sections came from the material that was pre-treated with ethanol.

Conclusion

Thus, we conclude that the use of KOH after leaf rehydration is unnecessary for anatomical studies of herbarium material. Its omission not only preserves the structural integrity of anatomical sections but also avoids the methodological, safety, and environmental drawbacks associated with the use and disposal of this reagent.

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