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Importance of anatomical leaf-blade features for the characterization of medicinal Commelinaceae in the Rio de la Plata area (Argentina)

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Summary: In this paper we examine the leaf-blade anatomy of: *Commelina erecta* L. var. *erecta* f. *erecta*, *Tradescantia fluminensis* Vell., *Tripogandra diuretica* (Mart.) Handlos, and *T. glandulosa* (Seub.) Rohweder. These species grow in the coastal region of the Río de la Plata (Argentina) where they are used as ophthalmic, diuretic, antirheumatic, sudorific, and digestive. The aim of this study was to establish diagnostic features in order to deepen knowledge of these medicinal species and thus, get a valuable tool to establish their botanical identity. The leaves were prepared according to usual methods for light and scanning electron microscopes. Histochemical techniques to identify starch, lipophilic substances, mucilage, tannins, and oxalate salts were performed. The ions analysis of oxalate salts using a scanning electron microscope equipped with an energy dispersive spectrometer was performed. The main differential traits found were: stomata hexaperiginous in *C. erecta* var. *erecta* f. *erecta*, and tetraperigenous in *T. fluminensis* and *Tripogandra* species; leaf blade margins with parenchyma in *C. erecta* var. *erecta* f. *erecta* and *T. fluminensis*, and with fibers in *Tripogandra* species; presence of druses in palisade parenchyma of the blade in *T. fluminensis*, and the hairy index.

Key words: Anatomy, *Commelina,* leaf-blade, medicinal plant, *Tradescantia, Tripogandra*.

Resumen: Importancia de la anatomía foliar en la caracterización de Commelinaceae medicinales en la región rioplatense (Argentina). En este trabajo se analiza la anatomía de la lámina foliar de: *Commelina erecta* L. var. *erecta* f. *erecta*, *Tradescantia fluminensis* Vell., *Tripogandra diuretica* (Mart.) Handlos y *T. glandulosa* (Seub.) Rohweder. Estas especies americanas, crecen en la región costanera del Río de la Plata (Argentina), donde son usadas como oftálmicas, diuréticas, antirreumáticas, sudoríficas y digestivas. El objetivo del estudio fue evaluar parámetros micrográficos foliares que permitan establecer caracteres diagnósticos con el fin de profundizar en el conocimiento de estas especies medicinales y obtener una herramienta para asegurar su identidad botánica. Las hojas fueron preparadas mediante técnicas usuales para microscopía óptica y microscopía electrónica de barrido. Mediante análisis histoquímicos se identificaron almidón, sustancias lipofílicas, mucílagos, taninos y sales de oxalato. Se realizó un análisis de los iones de las sales de oxalato con el uso de un microscopio electrónico de barrido, equipado con un espectrómetro de dispersión de energía. Las principales diferencias encontradas entre las especies fueron: estomas hexaperíginos en *C. erecta* var. *erecta* f. *erecta* y tetraperíginos en *T. fluminensis* y las especies de *Tripogandra*; margen de la lámina foliar con parénquima en *C. erecta* var. *erecta* f. *erecta* y *T. fluminensis* y con fibras en las especies de *Tripogandra*; drusas en el parénquima en empalizada de la lámina en *T. fluminensis* y el índice piloso.

Palabras clave: Anatomía, *Commelina,* hoja, planta medicinal, *Tradescantia, Tripogandra*.

Introduction

Commelinaceae comprises about 41 genera and 650 species (Faden, 1998). The genera are largely tropical and subtropical, but several of them extend to temperate regions of America, Africa, Asia, and Australia (Faden, 1983; Evans *et al*., 2000). In Argentina there are eight genera, three of them and nine species grow in the coastal region of Río de la Plata (Bacigalupo, 1984, 1996, Bacigalupo & Hurrell, 2008). This region is located in central-eastern of Argentina from the city of Diamante in Entre Ríos province to San Fernando in Buenos Aires province, and ending in the Río de la Plata. The studied species grow in lower delta area of

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the Paraná river, Martín García Island and Río de la Plata shore (cf. Fig. 1 in Novoa et al., 2012). The analized species are characterized by several features including a distinct closed leaf sheath and a succulent leaf blade (Fig. 1) (Cronquist, 1981; Faden, 1985; Faden & Hunt, 1991). They are perennial decumbent herbs, producing roots at nodes. They grow in shaded and humid places, and frequently are invasive species (Burns, 2004; Luque Arias & Estrada Sanchez, 2005). Ethnobotanical data have revealed that four species are used in folk medicine in the area under study (Lahitte *et al*., 2004; Hurrell *et al*., 2005). *Commelina erecta* var. *erecta* f. *erecta* contains mucilage which is used as ocular analgesic in conjunctivitis cases (Filipov, 1997; Martínez Crovetto, 1981; Rondina *et al*., 2008). The decoction of flowers and leaves are used to treat dermatitis and herpes, and the infusion for eye infections and hepatic disorders (Alonso Paz *et al*., 1995; Lahitte *et al*., 2004; Barboza *et al*., 2009). The plant is emollient and vulnerary (Alonso & Desmarchelier, 2005), and it has giardicidal biological activity, and the dried flowers are antibacterial (Barboza *et al*., 2009). The flowers of *Tradescantia fluminensis* are ophthalmic and dried aerial parts have biological aglucosidase activity (Barboza *et al*., 2009). The plant decoction of *Tripogandra diuretica* is externally used as antihaemorroidal, and drinked as diuretic, sudorific, antirehumatic and digestive (Lahitte *et al*., 2004; Hurrell *et al*., 2005; Bacigalupo & Hurrell, 2008). The flowers decoction of *Tripogandra glandulosa* is ophthalmic as well as *Tradescantia fluminensis* and *Tripogandra diuretica*. Based on the promising ethnopharmacological data on these four species, the aim of this contribution was to detect qualitative and quantitative micrographics leaf-blade features for the identification of this potential vegetal medicine regarding the botanical quality control.

Materials and Methods

Plant materials studied

Complete and fresh plants were collected in the Río de la Plata region, Argentina. One part of the fresh material was deposited in the (LPAG) herbarium, and another part was used to perform the study. For each species, leaves from specimens deposited in herbaria LP and LPAG (abbreviations according to Thiers, 2011) were also surveyed. Specimens vouchers data: ***Commelina erecta*** L. var. ***erecta*** f. ***erecta***: ARGENTINA. *Buenos Aires province*: *Pdo. La Plata*, La Plata, 6-XII-2011, *Arambarri 370* (LPAG); La Plata, Jardín Botánico y Arboretum "C. Spegazzini", Facultad de Ciencias Agrarias y Forestales, UNLP, 15-XII-2011, *Arambarri 450* (LPAG). ***Tradescantia fluminensis*** Vell.: ARGENTINA. *Buenos Aires province*: *Pdo. Ensenada*, Punta Lara, 28-X-1939, *Cabrera 5413* (LP); *Pdo. Tigre*, 17-X-1946, *Lanfranchi 582* (LP); *Pdo. La Plata*, La Plata, 12-XII-2014, *Arambarri 480* (LPAG). ***Tripogandra diuretica*** (Mart.) Handlos: ARGENTINA. *Buenos Aires province*: *Pdo. Berisso*, Balneario Blagiardi, 6-V-1995, *Bayón 308* (LPAG); *Pdo. La Plata*, Isla Martín García, 29-III-1997, *Hurrell, Belgrano, Jankowski* s.n. (LP); *Pdo. Tigre*, Delta, 4-IV-2009, *Hurrell, Ulibarri, Bazzano, Buet* 6844 (LP); ***Tripogandra glandulosa*** (Seub.) Rohweder: ARGENTINA. *Buenos Aires province*: *Pdo. La Plata*, La Plata, 3-I-1973, *Panella* s.n. (911 LPAG); *Pdo. La Plata*, Isla Martín García, 15-XII-1991, *Hurrell* 937 (LP).

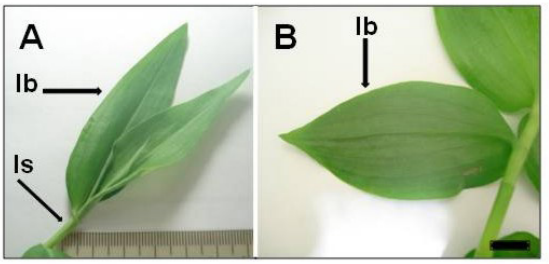

Fig. 1. Leaf parts. A: *Commelina erecta* var. *erecta* f. *erecta*. B: *Tradescantia fluminensis*. Detail of leaf-blade showing parallel venation. Scale bar: 1 cm. Abbreviations: leaf blade (lb); leaf sheath (ls).

Anatomical analyses

Fresh leaves samples were immediately fixed in formalin-glacial acetic acid-ethanol solution (FAA 70%) to avoid tissue alterations (Johansen, 1940). Dried leaf-blade parts from herbarium specimens were also surveyed. They were reconstituted by immersion in water in a closed pot, and placed in an oven at 30ºC for 24-72 h, and then fixed in FAA. Epidermis of leaf blade was obtained using the peeling off technique (D'Ambrogio de

Argüeso, 1986) or by transparenting leaf-blades with Dizeo de Strittmatter´s method (1973). To analyze the internal structure, the middle part of blade was transversally hand sectioned, bleached, in 50% sodium hypochlorite (NaOCl), washed three times with distilled water, and stained with 80% alcoholic solution of safranin or 1% aqueous solution of cresyl violet (D'Ambrogio de Argüeso, 1986) or alcian blue-safranin (Luque *et al.*, 1996), to distinguish different structures. Slides were mounted on glycerin jelly (Johansen, 1940).

Undulation of anticlinal epidermal cell walls, presence of waxes, stomata and hairs characteristics and distribution were examined at surface view. A quantitative analysis on eight areas in the half of both leaf blade surfaces was performed. The number of epidermal cells, stomata and hairs per field was determined; those cells located in the border of the field were not considered. The microscopic field used as the standard area was 560 µm in diameter. The average density of cells, stomata and hairs is expressed per square millimeter (d/mm2). Salisbury's stomata index was determined by means of the following equation: [no. of stomata / (no. of stomata + no. of epidermal cells)] x 100 (Stace, 1965). The hairy index was calculated using the same equation, changing no. stomata by no. hairs (Hernández *&* Albornoz, 2001). The two indexes are expressed in percentage (%).

Citochemical localization of cell components was performed with the following tests: iodine-potassium iodide (IKI) for starch (Ruzin, 1999), a saturated alcoholic solution of Sudan IV for lipophilic substances (Johansen, 1940), ferric chloride (10%) for tannins (Zarlavsky, 2014), cresyl blue (1%) (D'Ambrogio de Argüeso, 1986) and alcohol 100º (alcohol precipitate test) for mucilage (Korwar *et al*., 2010), and acetic acid (5%) to distinguish oxalate salts from carbonate (Yasue, 1969). Microscopic studies were performed using a Leitz SM Lux light microscope. The images were captured with a Moticam 1000 attached to the eyepiece of microscope, and Motic Image Plus 2.0 software. General terminology follows Metcalfe *&* Chalk (1979), and the ontogenetic classification of Fryns-Claessens *&* Van Cotthem (1973) is used. Nomenclature follows the International Plant Names Index (www.ipni.org) [Accessed: 2015/08/15].

An ions analysis of oxalate salts with a scanning electron microscope was performed. Portions of 0.5 cm^2 from the centre of FAA-fixed leaf blades were taken and critical point dried in CO_2. Then the samples were affixed on stubs by double-sided adhesive tape and they were submitted to metallization with a fine, thin gold layer by means of a cool diode sputter coating procedure. Afterwards they were examined with an environmental scanning electron microscope and energy dispersive spectrometer (ESEM-EDS) to determine the ionic nature of crystals.

Results

The main leaf-blade qualitative features are shown in Table 1, and the quantitative parameters are exhibited in Table 2.

Table 1. Qualitative leaf-blade microcharacters of four medicinal species of Commelinaceae.

Taxa	Leaf-blade type	Stomata type	Hairs on surfaces glandular	Hairs on surfaces non-glandular	Leaf-blade margin external	Leaf-blade margin internal	druses presence
Commelina erecta var. *erecta* f. *erecta*	amphistomatic	hexaperiginous	present	uncinulate	mucronate	parenchyma	absent
Tradescantia fluminensis	hypostomatic	tetraperiginous	absent	absent	short stiff hair	" "	present
Tripogandra diuretica	" "	" "	present	" "	" "	fibers	absent
Tripogandra glandulosa	" "	" "	" "	" "	" "	" "	" "

Table 2. Quantitative parameters of four medicinal species of Commelinaceae. Abbreviations: hairy index (HI); stomatal index (SI).

Taxa	Adaxial surface					Abaxial surface				
	Cells (d/mm2)	Stomata (d/mm2)	Hairs (d/mm2)	SI (%)	HI (%)	Cells (d/mm2)	Stomata (d/mm2)	Hairs (d/mm2)	SI (%)	HI (%)
Commelina erecta var. *erecta* f. *erecta*	462.12	18.94	15.15	3.94	3.17	579.54	49.24	26.51	7.83	4.35
Tradescantia fluminensis	106.06	0	0	0	0	344.71	37.88	0	9.91	0
Tripogandra diuretica	335.22	0	9.46	0	2.73	691.28	73.86	17.04	9.64	2.41
Tripogandra glandulosa	125.01	0	0	0	0	417.04	42.42	9.46	9.23	2.21

Epidermal characteristics in surface view

Epidermal cells are polygonal in shape with straight to curved anticlinal cell-wall patterns (Fig. 2A-C). Outer periclinal cell walls present wax ornamentation with granulate appearance, and they are conspicuously marked on surfaces of *Tripogandra* species leaves (Fig. 2A).

Stomata are distributed on both faces in *C. erecta* var. *erecta* f. *erecta*, whilts are found exclusively on the abaxial side in *Tradescantia* and *Tripogandra* species (Tab. 1). Stomata in *Tradescantia* and *Tripogandra* species are tetraperigenous (Tab. 1; Fig. 2A, B). The tetraperigenous adult stoma has two lateral subsidiaries cells as long as guard cells, and the two polar ones as broad as the stomatal complex in the species analyzed. The two lateral subsidiaries cells have deltoid shape in both *Tripogandra* species (Fig. 2A), whereas *T. fluminensis* has rectangular subsidiaries lateral cells (Fig. 2B). In *Commelina* we found an hexaperigenous stomatal complex (Tab. 1; Fig. 2C), with an arrangement of these 6-celled (hexacytic) adult stomata with the second pair of lateral subsidiaries as long as the entire stomatal complex. One common trait in the stomata of the studied species is that the guard cells have T shaped cuticle thickening in the polar region (Fig. 2A-C).

Glandular and non-glandular hairs are found in *Commelina* and *Tripogandra* and only non-glandular in *Tradescantia*. The glandular ones have a short basal cell, another cutinized cell and a tenuous, oblong and obtuse apical cell. They are present on leaf-blade surfaces of *Commelina* and *Tripogandra* (Tabs. 1 and 2). The non-glandular hairs are simple, two-three cellular and straight or with the apical cell incurved (uncinate or hooked). The hooked hairs are only found on the surfaces of *C. erecta* var. *erecta* f. *erecta* leaf-blade (Tab. 1, Fig. 2D). *Tradescantia fluminensis* and the two studied species of *Tripogandra*, have short stiff, apically directed hairs on the leaf-blade margins (Tab. 1; Fig. 2E), however we found that these hairs are absent in *C. erecta* var. *erecta* f. *erecta* which presents a mucronate leaf-blade margin (Tab. 1; Fig. 2F).

Leaf-blade cross section

The two epidermis are formed by a single-layer of thin-walled quadrangular or rectangular cells, elongated perpendicularly to the surface. The adaxial epidermis layer comprises more than half of the thickness of the leaf (Fig. 3A). Stomata in cross section are located at the same level as the remaining epidermal cells (Fig. 3B).

The mesophyll is heterogeneous, formed by one layer of palisade parenchyma towards the adaxial surface, and two-three layers of spongy parenchyma towards the abaxial surface (Fig. 3C, D). Spongy parenchyma is formed by arm cells and conspicuous intercellular spaces (Fig. 3C). *Tradescantia fluminensis* differs because the layer in contact with the palisade is formed by rounded cells, and arm cells are found in the layers towards the abaxial side (Fig. 3D). The studied species present a midrib cross-section concave on the adaxial side and slightly convex on the

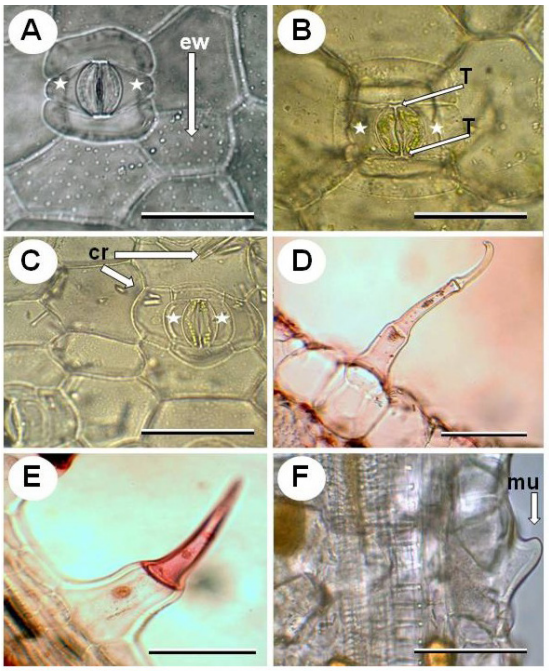

Fig. 2. Epidermis in surface view: A-C. A, *Tripogandra glandulosa*: epidermis showing anticlinal straight cell walls, and epicuticular waxes on the surface; tetraperigenous stomata complex with deltoid lateral subsidiary cells. B, *Tradescantia fluminensis*: tetraperigenous stomata complex with rectangular lateral subsidiary cells. C, *Commelina erecta* var. *erecta* f. *erecta*: abaxial epidermis with curved anticlinal cell walls, inconspicuous waxes granules on the surface, crystals contained in epidermal cells, and hexaperigenous stomata complex. In A and B, stars indicate the two lateral subsidiary cells, and in C, the first of the two pairs of lateral subsidiary cells. In A, B, C, polar cuticle thickening T-shaped are seen on the guard cells. Hairs and mucronate margin in leaf-blade cross sections: D, hooked hair on abaxial surface in *Commelina erecta* var. *erecta* f. *erecta*. E, short stiff hair on leaf-blade margin with acute, and cutinized apical cell in *Tripogandra glandulosa*. F, mucron on the leaf-blade margin in *Commelina erecta* var. *erecta* f. *erecta*. Scale bars = A-C, F: 100 µm; D, E: 150 µm. Abbreviations: crystals (cr); epicuticular wax (ew); mucron (mu); polar cuticle thickening T-shaped (T).

opposite side. The epidermis is uniseriate, and the parenchyma is found below the adaxial side (Fig. 3E), or sometimes one-two layers of the suberized cell walls are adjacent to both epidermis (Fig. 3F). The midrib has one small collateral vascular bundle surrounded by a parenchyma sheath and it is embedded in the parenchyma tissue (Fig. 3E, F).

The leaf-blade margin showed interesting differences among studied species. Externally, the leaf margin is mucronate in *C. erecta* var. *erecta* f. *erecta* (Tab. 1; Fig. 4A, B) whereas *T. fluminensis*, *T. diuretica,* and *T. glandulosa* exhibit short stiff hairs (Tab. 1; Fig. 4C-F). The internal structure of the leaf-blade margin in *C. erecta* var. *erecta* f. *erecta* shows parenchyma and a vascular bundle with a large tracheal diameter (Fig. 4A, B). *Tradescantia fluminensis* presents parenchyma with a vascular bundle without a large tracheal diameter (Fig. 4C, D), and the two species of *Tripogandra* exhibit more than five layers of fibers before reaching the parenchyma area and the vascular bundle (Tab. 1; Fig. 4E, F).

Crystals are frequent in the family. Raphides sacs are arranged in longitudinal series parallel to the veins (Fig. 4C). In the mesophyll there are also calcium oxalate crystals, solitaries and macles (Fig. 3E; Fig. 5A), and these are also found in the epidermal cells of *C. erecta* var. *erecta* f. *erecta* (Fig. 2C). *Tradescantia fluminensis* is the only species that shows druses distributed in the palisade parenchyma (Tab. 1; Fig. 3D, E). It was determined that all crystal types are of oxalate salts with the ion calcium (Fig. 5A, B). Starch, tannins and mucilage compounds were also found in the mesophyll (Fig. 3E; Fig. 4A).

Discussion

Epidermal characteristics in surface view. All species studied showed epicuticular waxes of granular appearance on the epidermal outer periclinal cell walls. This epicuticular wax type agrees with classification and terminology proposed by Barthlott *et al.* (1998). The distribution of stomata on both surfaces in *C. erecta* var. *erecta* f. *erecta,* and only on the abaxial epidermis in *T. fluminensis* and both *Tripogandra* species are in agreement with data reported for *Commelina* by Elb *et al*. (2010), and in *Tradescantia* by Croxdale (1998). Concerning the stomata pattern we found tetraperigenous and hexaperigenous types. The tetraperigenous have been named tetracytic by Metcalfe *&* Chalk (1950), and reported for *Tradescantia* by Strasburger (1866), but never cited

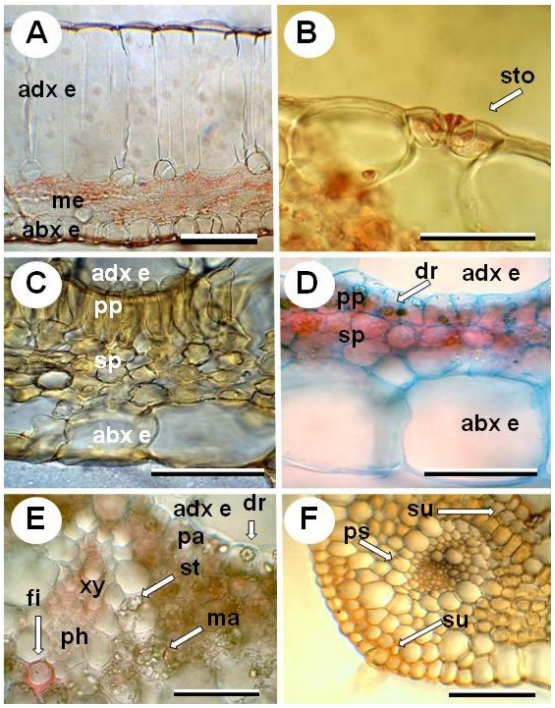

Fig. 3. Leaf-blade cross sections. A, *Tripogandra glandulosa*: adaxial epidermis one layered with rectangular cells conspicuously elongated perpendicularly to the surface of the leaf, heterogeneous mesophyll, and rounded cells in the abaxial epidermis. B, Stoma located at the same level as the remaining epidermal cells. C, *Commelina erecta* var. *erecta* f. *erecta*: adaxial epidermis, dorsiventral mesophyll with palisade parenchyma one layered, and spongy parenchyma formed by three layers of arm cells and conspicuous intercellular spaces adjacent to abaxial epidermis. D, E, *Tradescantia fluminensis*: D, adaxial epidermis, dorsiventral mesophyll showing quadrangular cells of palisade parenchyma containing druses, and spongy parenchyma two layered, one layer with rounded cells in contact with the palisade one, and a layer with notable intercellular spaces adjacent to abaxial epidermis; E, cross section of the midrib showing adaxial epidermis, parenchyma containing druses, one collateral vascular bundle [xylem, phloem, and fiber], encircled by a parenchyma sheath, embedded in the parenchyma with starch and macles. F, *Commelina erecta* var. *erecta* f. *erecta*: cross section of the midrib showing one layer of suberized cell walls adjacent to both epidermis, the collateral vascular bundle encircled by a conspicuous parenchyma sheath. Scale bars = A: 300 µm; B-E: 100 µm; F: 150 µm. Abbreviations: abaxial epidermis (abx e); adaxial epidermis (adx e); druses (dr); fiber (fi); macles (ma); mesophyll (me); parenchyma (pa); phloem (ph); palisade parenchyma (pp); parenchyma sheat (ps); spongy parenchyma (sp); starch (st); stoma (sto); suberized cell walls (su); xylem (xy).

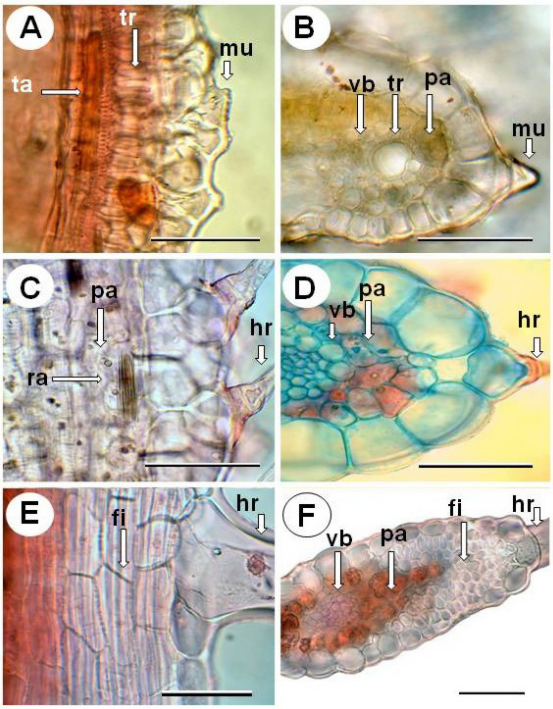

Fig. 4. Leaf-blade margin. A, C, E, longitudinal sections, and B, D, F, cross sections. A, B, *Commelina erecta* var. *erecta* f. *erecta*: A, mucron on the leaf-blade margin, parenchyma and the vascular bundle with the presence of a large tracheal diameter; tannins are found; B, mucron, parenchyma, large tracheal diameter, vascular bundle. C, D, *Tradescantia fluminensis*: C, non-glandular hairs on the leaf-blade margin, parenchyma, and one vascular bundle without a large tracheal diameter; raphides are found; D, hair on the margin, parenchyma and vascular bundle. E, F, *Tripogandra* spp.: E, non-glandular hairs on the leaf-blade margin, more than five layers of fibers before to reach the parenchyma and vascular bundle; F, hair on the margin, numerous layers of fibers, parenchyma, vascular bundle. Scale bars: A-F: 100 µm. Abbreviations: fibers (fi); non-glandular hairs (hr); mucron (mu); parenchyma (pa); raphides (ra); tannins (ta); large traqueal diameter (tr); vascular bundle (vb).

for *Tripogandra*. The arrangement of the 6-celled hexaperigenous adult stomata was reported in Commelinaceae family by Tomlinson (1969). The differences in stomata complex of *Tradescantia* and *Tripogandra* regarding *Commelina* have a

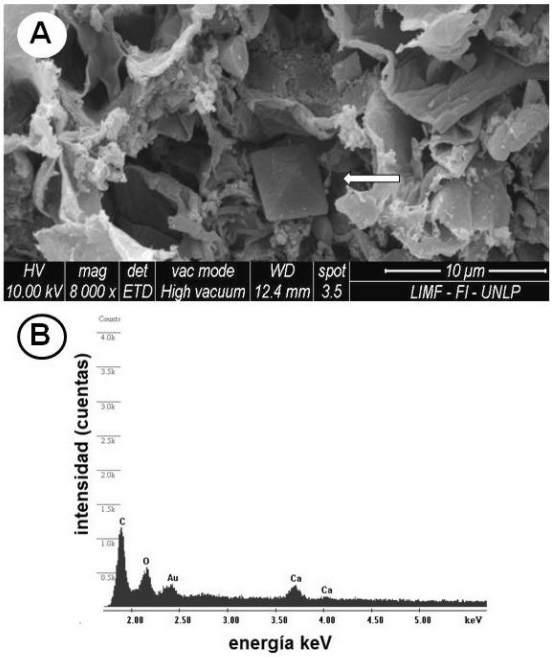

Fig. 5. Analysis of oxalate salts by ESEM-EDS. A: photograph of one macle (indicated by arrow). B: ion calcium spectrum.

taxonomic value, because it coincides with the classification of Commelinaceae (Faden *&* Hunt, 1991) since *Tradescantia* and *Tripogandra* belong to the tribe Tradescantieae whereas *Commelina* belongs to the tribe Commelineae.

Glandular and non-glandular hairs were described and illustrated previously on the stem epidermis of *C. erecta* var. *erecta* f. *erecta* by Novoa et al. (2012). According to Faden (1998), the simple non-glandular hairs, two-three cellular and straight with an acute apex are common in the family, however we found hooked hairs on the surfaces and a mucronate leaf-blade margin in *C. erecta* var. *erecta* f. *erecta.*

Leaf-blade cross section. The large epidermal cells with thin walls are a water-storage tissue, and due to this fact it was described as a succulent surface layer by Solereder *&* Meyer (1929). These species are mesophytic, this condition is corroborated by the stomata located at the same level of epidermal cells. It coincides with observation reported by Elb *et al.* (2010).

Our found of raphides sacs arranged in longitudinal series parallel to the veins is in agreement with Faden (1998). However we also found solitaries, macles, and druses calcium oxalate crystals in epidermis and mesophyll. The druses only in the palisade parenchyma of *Tradescantia fluminensis*. Starch, tannins and mucilage compounds were found in the mesophyll, and Barboza et al. (2009) also reported steroids and oxidases in plants of *C. erecta* var. *erecta* f. *erecta.*

In summary this comparative study has a broad potential to improve our understanding to identify species in entire samples or powered states, and to ensure a botanical quality control. The main qualitative features to distinguish genera were stomata, hairs, and crystal types and distribution, and the external and internal traits of leaf-blade margins. The hairy index parameter contributed to distinguish the analyzed species.

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