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Original research

Histochemical localization of urushiols in stems and leaflets of *Schinopsis lorentzii* and *S. marginata* using diazonium salts

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ABSTRACT

The trees *Schinopsis lorentzii* and *S. marginata* produce alk(en)ylcatechols, also known as urushiols, which function in plant defense as antifungals, antifeedants and cause contact dermatitis in human beings. Here, we propose the use of the diazonium salts Fast Blue B ½$ZnCl_2$ and Fast Blue RR $ZnCl_2$ for the *in situ* histochemical localization of urushiols in stems and leaflets of *S. lorentzii* and *S. marginata*. The main surface and internal anatomical features of the aerial plant parts were visualized with cresyl violet and astra blue safranin. Extracts from leaflets and stems were separated by TLC and revealed with aqueous solutions of 0.5% Fast Blue B ½$ZnCl_2$ in 5% acetic acid (FBB), 0.5% Fast Blue RR $ZnCl_2$ in 5% KOH (FBRR) and 5% $FeCl_3$. Fresh cross sections of leaflets and stems were also stained with these chromogenic reagents. Mature leaflets and stems showed few glandular trichomes in their surfaces. Well developed phloem schizogenous ducts were uniformly distributed in the leaflet mesophylls and radially organized in the cross sections of the stems. TLC of the extracts revealed with FBB, FBRR and 5% $FeCl_3$ gave bluish black spots for urushiols. Very few constituents of the extracts other than the urushiols revealed the same colour after spraying with the diazonium salts. FBB and FBRR indicated the presence of urushiols in both the cavity of the phloem schizogenous ducts and their surrounding epithelial cells. Urushiols were not visualized neither in the cuticle of the aerial parts nor in the glandular trichomes. FBB was coupled to more individual polyphenol species than FBRR whereas $FeCl_3$ was by far a less specific chromogenic reagent. Nevertheless, FBRR was more selective towards the urushiols than FBB. The exposure to urushiols should be possible only after the breakdown of leaflets and stems of *Schinopsis* trees.

1. Introduction

The genus *Schinopsis* (tribe Rhoeae, Anacardiaceae family) comprises seven neotropical species native from South America (Pell et al., 2011). In northwest Argentina, it is represented by *S. lorentzii* and *S. marginata*. They are timber species used for making buildings and railroad ties (Barberis et al., 2012). Rural workers involved in the exploitation of these trees often suffer contact dermatitis which is due to the presence of alk(en)ylcatechols in the aerial parts (Aristimuño Ficoseco et al., 2014). These constituents, also known as urushiols, likely have a phytoanticipin role *in vivo*. They showed antifungal activity against fungi of the genus *Fusarium* (Aristimuño Ficoseco et al., 2017), *Alternaria* (Harborne, 1999) and *Helmintosporium* (Anaya et al., 1999), and act as deterrents of hemiptera insects (Aguilar-Ortigosa and Sosa, 2004; Suchan and Alvarez, 2015). Current findings suggest that in the Rhoeae tribe the tissular localization of urushiols can be species specific. Urushiols were reported in the sap fluids of the phloem ducts in leaves of *Toxicodendron* species (*T. radicans*, *T. vernicifluum* and *T. diversilobum*) while they were found in the epicuticular leaf layers of *Lithraea caustica* (Urzúa et al., 2011; Zhao et al., 2014). *Toxicodendron* species must release urushiols after crushing the aerial parts of the plant while in *L. caustica* a direct exposition occurs after contact with the intact stems or leaves (Urzúa et al., 2011). The location of urushiols in the tissues of aerial parts of the *Schinopsis* trees and the resulting risk of exposure has not been investigated so far.

A number of elegant approaches have been developed for the *in situ* localization of alk(en)ylcatechols in *Toxicodendron* species. They include the use of matrix-assisted laser desorption/ionization-mass spectrometry imaging (MALDI-MSI) and fluorescence detection of urushiol-derivatives generated after reaction with Bn-butylboronic acid (Aziz et al., 2017; Braslau et al., 2013). These approaches, however, require the access to expensive equipments or important management and

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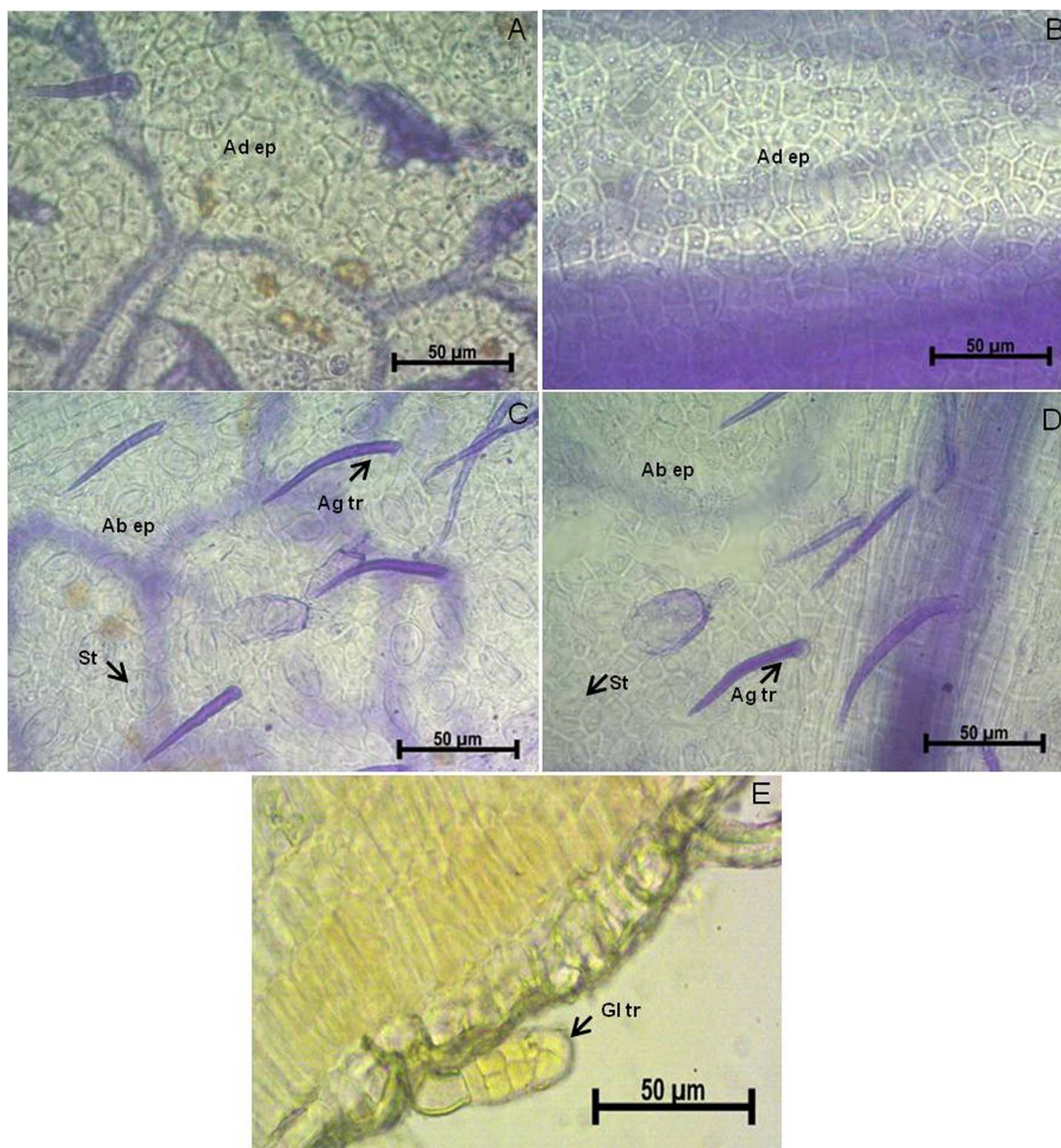

Fig. 1. Paradermal views of the leaflet epidermis of the *Schinopsis* trees stained with cresyl violet. The adaxial side is visualized for (A) *S. lorentzii* and (B) *S. marginata*. The abaxial side is observed in (C) and (D) for the mentioned species, respectively. A glandular trichome (E) is also shown. Ep ad, adaxial epidermis; Ep ab, abaxial epidermis; Es, stomata, Tr eg, aglandular trichome; Tr gl, glandular trichome.

expertise in the preparation of specific reagents. An attractive alternative could be the *in situ* generation of azo dyes after coupling the urushiols with diazonium salts. Colorimetric methods based on the use of these salts were developed for the quantification of both urushiols and alk(en)ylresorcinols in plant extracts (Sampietro et al., 2009, 2013a,b; Landberg et al., 2008). They were also succesfully used for the visualization of urushiols in thin layer chromatography (Aristimuño Ficoseco et al., 2014). The aim of this work was to test the use of the diazonium salts Fast Blue B ½$ZnCl_2$ and Fast Blue RR $ZnCl_2$ as *in situ* staining reagents of urushiols in leaves and stems of *S. lorentzii* and *S. marginata*.

2. Materials and methods

2.1. Plant material

Fresh leaves and stems of *Schinopsis marginata* and *S. lorentzii* were collected during February –March of 2014 from trees located near the national road 9 (26° 31′ 33.45” south latitude, 65° 18′ 16.24” west longitude), 45 km north of the city of San Miguel de Tucuman. Three fresh samples were obtained per tree, each one at the middle of the tree top. Three trees belonging to each tree species were sampled. The botanical identity of the plant materials was confirmed by Dr. Nora Muruaga (Laboratory of taxonomy of phanerogamic plants, Miguel Lillo Foundation, Tucuman, Argentina) by comparison with voucher specimens already deposited in the Herbarium of the Miguel Lillo Foundation.

2.2. Preparation of plant extracts and phytochemical analysis

Leaves of *S. lorentzii* and *S. marginata* were separately powdered in a Wiley mill. A portion of the powdered material (45 g) was sequentially extracted with solvents of increasing polarity (dicloromethane, ethyl acetate and methanol). The powdered plant material was macerated for

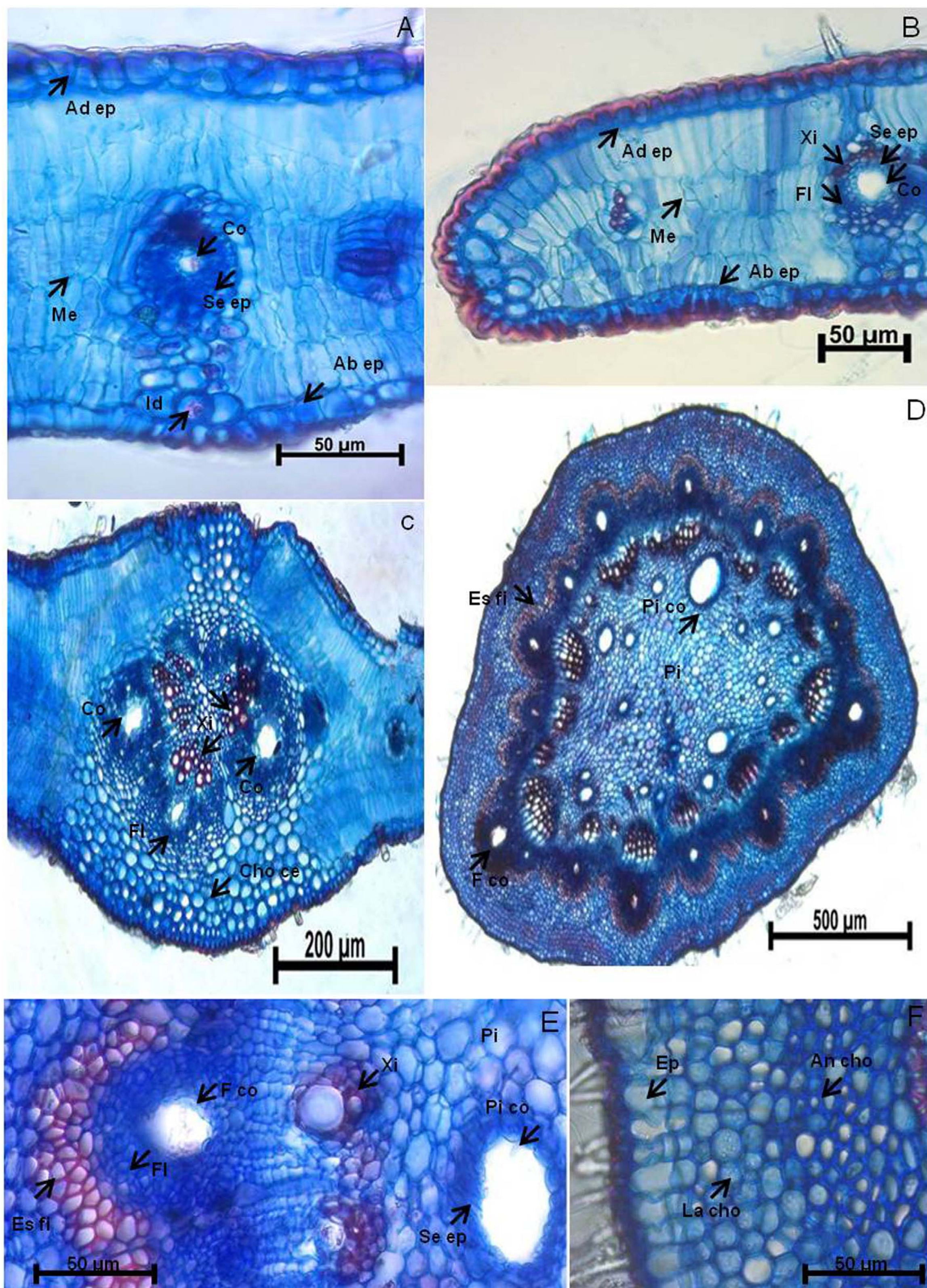

Fig. 2. Cross sections of leaflets and stems stained with safranin–astra blue. Leaflets of *S. lorentzii* (A) at the level of a secondary nerve, and (C) showing the midrib. Leaflet of *S. marginata* (B) at the border showing a secondary nerve. Stem of *S. marginata*: (D) general view of a cross section, partial views showing (E) the schizoneous ducts in the phloem and the pith, and (F) the angular and laminar chollenchyma. Ep, epidermis; Ep ad, adaxial epidermis; Ep ab, abaxial epidermis; Me, mesophyll; Co, schizogenous conduct; Xi, xylem; Fl, phloem; Ep se, secretory epithelium; Ce pa, parenchymal cells; Ce co, chollenchymal cells; Id, idioblasts. Med, pith; Fi es, sclerenchymatous fibers; Co es, schizogenous secretory ducts; Ep se, secretory epithelial cells; Co la, laminar chollenchyma; Co an, angular cholenchyma, Cf, phloematic conduct; Cm, pith conduct.

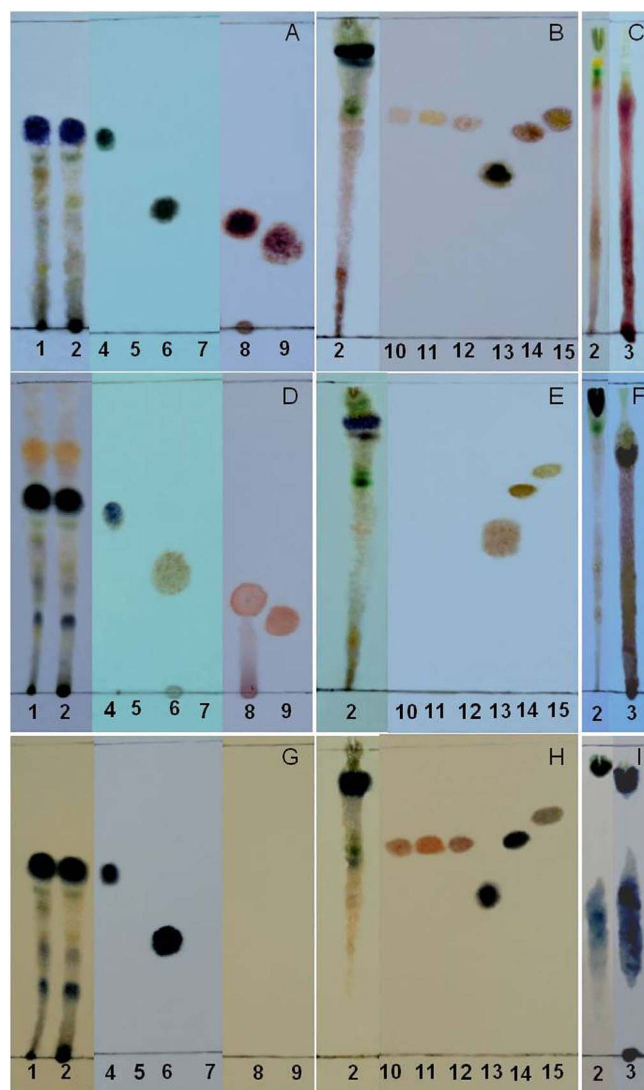

Fig. 3. TLC chromatograms of (1) the dichloromethane, (2) the ethyl acetate, and (3) the methanolic extracts of *S. lorentzii*; (4) heptadecylcatechol; (5) lupeol; (6) catechol; (7) ergosterol; (8) olivetol; (9) orcinol; (10) vanillic acid; (11) *p*-coumaric acid; (12) ferulic acid; (13) gallic acid; (14) quercetin; and (15) kaempferol. Plates (A, B, C) were stained with aqueous solutions of acidic 0.5% Fast Blue B; plates (D, E, F) with basic 0.5% Fast Blue RR; plates (G, H, I) with 5% $FeCl_3$. The mobiles phases were: (A, D, G) hexane: ethyl acetate (7:3, v/v); (B, E, H) toluene: ethyl acetate: formic acid (4:5:1, v/v/v); (C, F, I) toluene: ethyl acetate: formic acid (3:10:1, v/v/v).

24 h with each organic solvent (1:4.4, w/v), filtered using a filter paper. The extracted plant material was left to dry at room temperature before immersion in the next organic solvent. The organic extracts thus obtained were concentrated under reduced pressure and used for thin layer chromatrography analysis (TLC).

The presence of urushiols in the extracts was evaluated by TLC on silica gel 60 F254 plates developed with mobile phase 1 (hexane:ethyl acetate, 7:3, v/v), mobile phase 2 (toluene: ethyl acetate: formic acid 4:5:1, v/v/v) or mobile phase 3 (toluene: ethyl acetate: formic acid, 3:10:1, v/v/v). The chromatograms were sprayed with aqueous solutions of 5% $FeCl_3$, 0.5% Fast Blue B $ZnCl_2$ in 5% acetic acid (FBB) or 0.5% Fast Blue RR ½$ZnCl_2$ in 5% KOH (FBRR). Putative polyphenols previously reported in Anacardiaceae species were also subjected to TLC analysis. They were heptadecylcatechol, catechol, olivetol, orcinol, quercetin, kaempferol, gallic acid, vanillic acid, ferulic acid and *p*-coumaric acid purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Anatomical analysis

Ten leaves and ten stem segments were selected from each *Schinopsis* species. Two leaflets were detached from each leaf, and two herbaceous fragments were obtained from each stem. Then, the plant material was fixed in FAA (100 ml of 37% formaldehyde, 300 ml of 96% ethanol, 50 ml of 100% glacial acetic acid and 35 ml of distilled water). Medium and terminal zones of both leaflets and stem fragments were sectioned with a Thermo Scientific™ HM 325 Rotary Microtome (thickness range: 5–25 μm). The cuts were cleared with 50% sodium hypochlorite and then coloured in two successive steps with astra safranin-blue. Epidermal tissues were diaphonized following the technique of Dizeo de Strittmatter (1973). The leaflet or stem sections were left a week in 5% KOH till complete loss of cell contents. Clearing was completed in water:sodium hypochlorite, 1:1 (v/v). The diaphonized material was coloured with a simple metachromatic staining in cresyl violet.

2.4. Histochemical tests

Fresh stems and leaflets were sectioned with the rotary microtome. Then, they were subjected to $FeCl_3$, FBB and FBRR. These chromogenic reagents were applied at a rate of one drop per leaf or stem section. The contact time between a chromogenic solution and a histological section was 5 min for FBB or FBRR and 1 min for $FeCl_3$. The excess of chromogenic solution was removed from the leaf or the stem section by three successive washes with distilled water. The coloured sections were mounted in distilled water and observed under the optic microscope.

2.5. Microscopic analysis of coloured plant tissues

The coloured sections of the plant tissues were observed in an optical microscope Karl Zeiss Axiostar plus. The pictures were taken with a digital camera Axio Cam ERc 5S Zeiss.

3. Results

The surface anatomical characters of the plant organs were visualized with cresyl violet which coloured dark blue the lignin and tan violet the cellulose of the cell walls. Both stems and leaflets showed a papilose epidermis of orthogonal cells with straight anticlinal walls, ciclocytic stomata and a thick striated cuticle (Fig. 1A and B). The leaflets were amphistomatic with the highest stomatal density located at the abaxial side (854 stomata/mm^2, *S. lorentzii*; 564 stomata/mm^2, *S. marginata*). Unicellular non-glandular trichomes were observed on both stems and leaflets (Fig. 1C and D). They were more abundant in the abaxial leaflet surface (45 cells/mm2, *S. lorentzii*; 28 cells/mm^2, *S. marginata*) than in the adaxial epidermis (32 cells/mm^2, *S. lorentzii*; 15 cells/mm^2, *S. marginata*). Few glandular trichomes with an unicellular foot and a pluricellular head of 5–7 cells were found in the leaflet bases, near to the midribs and the main nerves (Fig. 1E). Regarding the internal anatomy, fresh cross sections of the leaflets and stems were observed after dying with safranin-astra blue. This reagent stained blue the cell walls rich in cellulose and dark pink to red the walls rich in lignin or suberin. In this way, it was possible to see the cellulose rich walls of palisade parenchyma cells forming a uniform mesophyll which was interrupted by bicolateral vascular bundles. The last ones were delimited by a sheat of parenchymal cells and were formed by poles of lignified xylematic vessels surrounded by phloem poles (Fig. 2A and B). Schizogen ducts bordered by a multilayered sheat of epithelial parenchyma cells interrupted the phloem poles. They appeared in numbers of three to four at the leaflet midribs and at regular intervals in the cross sections of the leaflets as part of the vascular bundles (Fig. 2C). Beneath the epidermis, the stems showed two layers of angular and laminar collenchyma cells, followed in depth by a 10–20 cell-layered

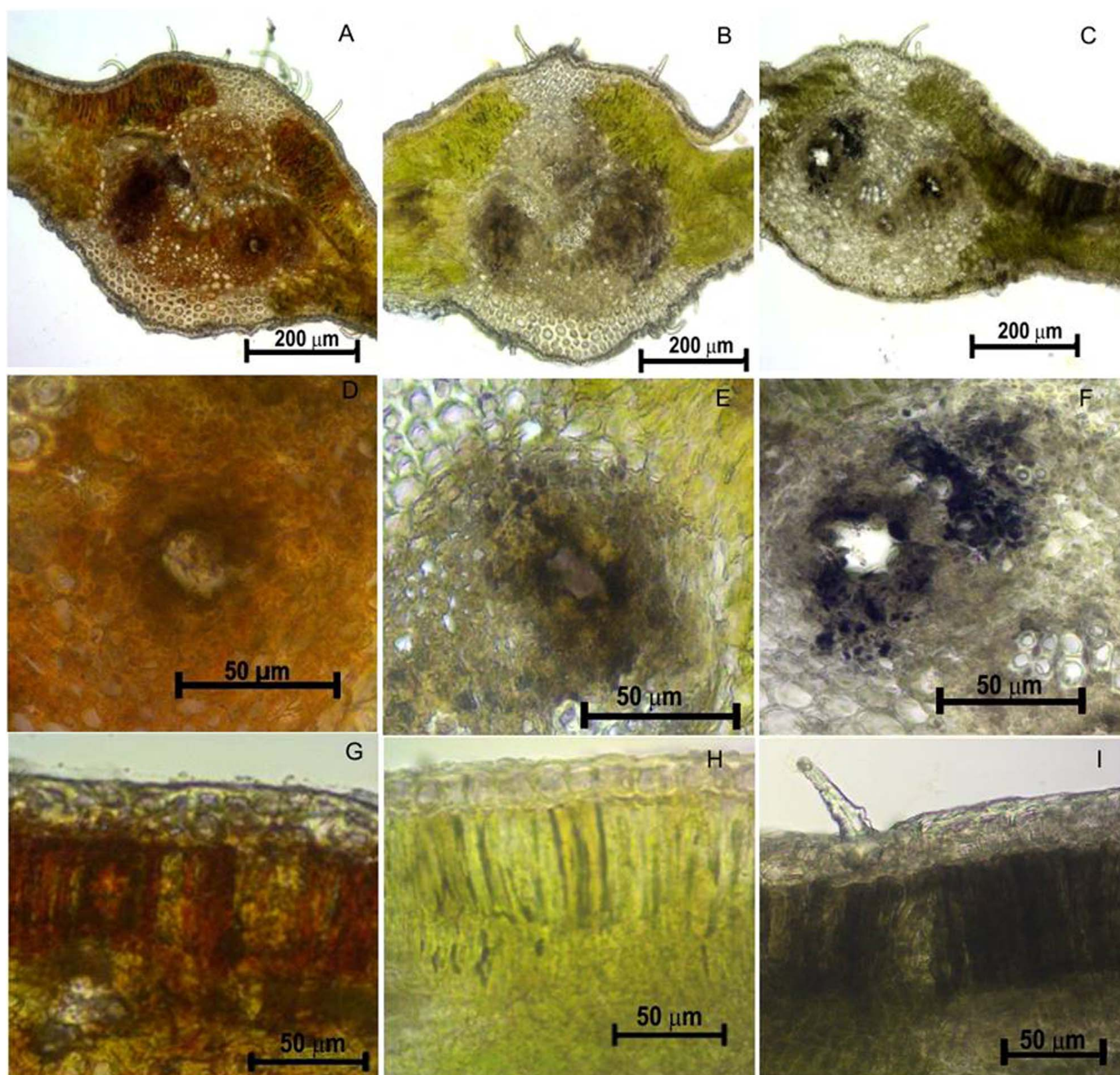

Fig. 4. Cross sections of leaflets from *S. lorentzii* (A, D, G) and *S. marginata* (B, C, E, F, H, I) stained with aqueous solutions of Fast Blue B (A,D,G), Fast Blue RR (B,E,H) and $FeCl_3$ (C,E,I). Cuts are observed at the level of the midrib (A,B,C), a phloem schizogenous duct (D,E,F), and the top layer of the palisade parenchyma cells of the mesophyll (G,H,I).

parenchymatic cortex (Fig. 2F). The central cylinder had 20–25 open collateral bundles with sclerenchymatous cap fibers. Sieve tubes of the phloem and their companion cells were observed as a continuous dark blue ring. The stems also had phloematic schizogenous ducts which followed the radial distribution of the vascular bundles. Idioblasts with druses were found in wide parenchymatic pith interrupted by circular schyzogenous ducts of different sizes (Fig. 2D and E).

Thin layer chromatographies of the leaflet and stem extracts were sprayed with the aqueous solutions FBB, FBRR and $FeCl_3$. The results are presented only for the leaflet extracts of *S. lorentzii* (Fig. 3) because the pattern and colours of bands for these extracts was the same as that visualized for the stem and leaflet extracts of *S. marginata* and the stem extracts of *S. lorentzii*. The dichloromethane and ethyl acetate extracts developed with mobile phase 1 showed the same TLC profile of 8 spots (bluish black, Rf = 0.68; green, Rf = 0.56 and 0.47; brown, Rf = 0.53 and 0.20; dark brown, 0.44; bluish green, Rf = 0.36; gray, Rf = 0.15); 8 spots (orange, Rf = 1, 0.91 and 0.82; bluish black, Rf = 0.68; green, Rf = 0.56; dark gray, Rf = 0.53, 0.44, 0.36 and 0.27) and 5 spots (bluish black, Rf = 0.68, 0.56, 0.53, 0.44 and 0.27) after application of FBB, FBRR and $FeCl_3$, respectively (Fig. 3A, D and G). The most polar constituents of the ethyl acetate extracts were separated using mobile phase 2 (Fig. 3B, E and H). Spots visualized were 8 with FBB (green, Rf = 0.97 and 0.75; bluish black, Rf = 0.91; dark grey, Rf = 0.87; reddish brown, Rf = 0.71, 0.66, 0.23 and 0.11), 7 with FBRR (green, Rf = 0.94, 0.73 and 0.69; dark blue, Rf = 0.88; dark gray, Rf = 0.84; brown, Rf = 0.53 and 0.20) and 6 with $FeCl_3$ (bluish black, Rf = 0.91; green, Rf = 0.72, 0.68 and 0.64; brown, Rf = 0.58 and 0.53). The TLC of the methanolic extracts showed a poorly resolved and fuzzy pattern of spots that were brown (FBB and FBRR) or bluish black ($FeCl_3$) (Fig. 3C, F and I). Thin layer chromatographies of the individual polyphenol species were also sprayed (Fig. 3A, B, D, E, G and H). Spots of these phenolic compounds were coloured bluish blue (FBB and $FeCl_3$ on catechol, heptadecylcatechol and gallic acid; $FeCl_3$ on quercetin; FBRR on heptadecylcatechol), red (FBB and $FeCl_3$ on *p*-coumaric, ferulic and vanillic acids; FBB on olivetol, orcinol, quercetin, kaempferol), dark grey (FBRR on catechol; $FeCl_3$ and FBRR on kaempferol)

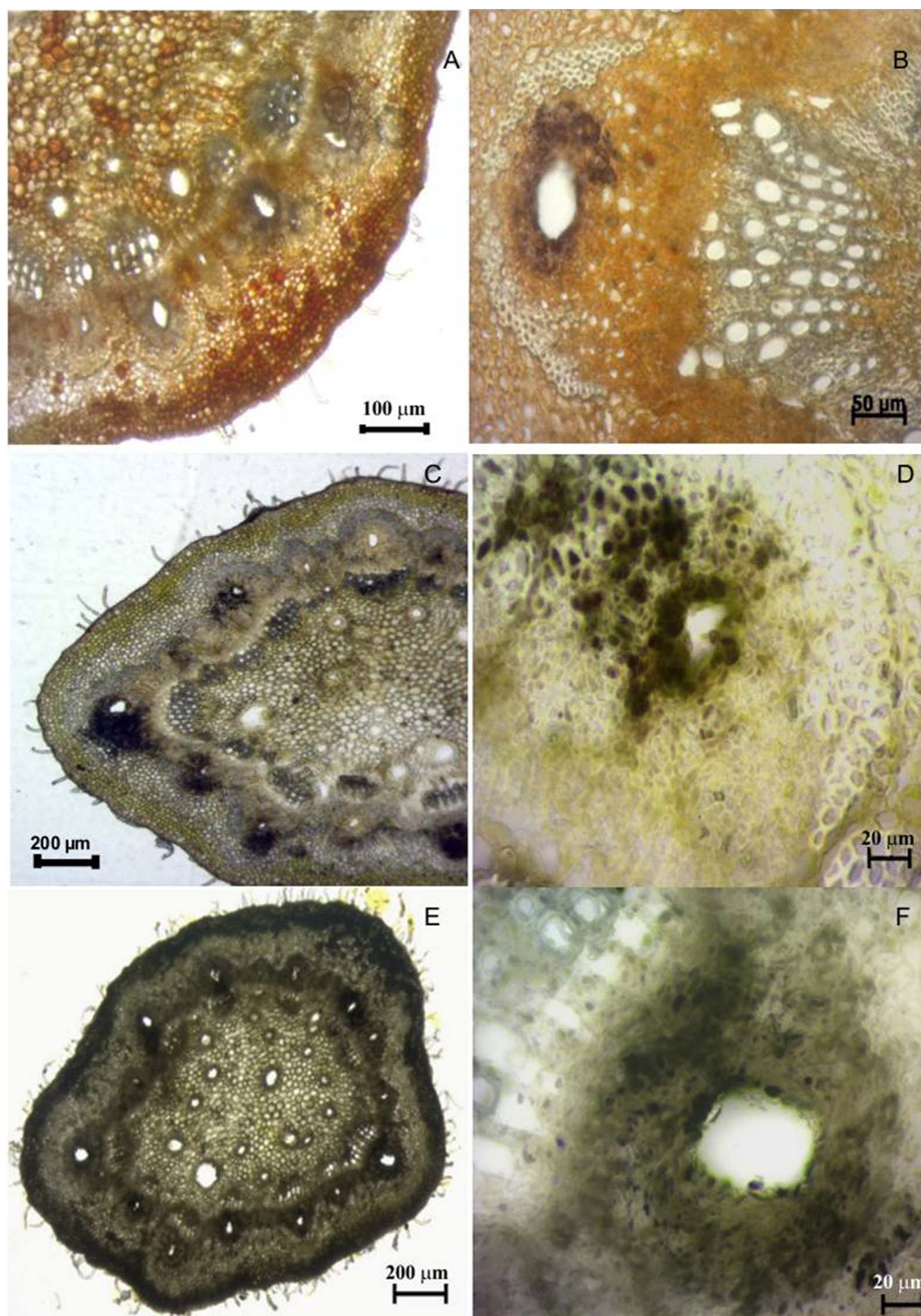

Fig. 5. Partial cross sections of *S. lorentzii* stained with aqueous solutions of (A,B) acidic Fast Blue B at 0.5% and (E,F) aqueous $FeCl_3$; and of *S. marginata* stained with (C,D) aqueous basic Fast Blue RR at 0.5%.

and brown (FBRR on quercetin and gallic acid).

The diazonium salts and $FeCl_3$ stained grey the lignified cells of the xylem and the fibers, the cuticle and the epidermal cells of the leaflets including the glandular and non-glandular trichomes (Fig. 4A, B and C). The mesophyll cells appeared brown (FBB) or bluish black ($FeCl_3$). The highest intensity of these colours was observed in the cell layers located towards the upper epidermis of the leaflets (Fig. 4G and I). The mesophyll cells were not stained by FBRR (Fig. 4H). The schizogenous ducts and their surrounding epithelial cells appeared with a dark-reddish brown (FBB) or a dark-bluish dark colour (FBRR or $FeCl_3$) (Fig. 4D, E and F). In the cross sections of the stems, the collenchymatic tissue was observed reddish-brown (FBB), green-gray (FBRR) or bluish black ($FeCl_3$), while the cortex cells were visualized light brown (FBB), grey (FBRR) or bluish grey ($FeCl_3$) (Fig. 5A, C and E). The staining reagents tinted gray the xylematic and fiber cells. The phloematic cells were reddish-brown (FBB) or light grey (FBRR) with the exception of the epithelial cells bordering the schizogenous ducts which were coloured brown-red (FBB), or dark black (FBB and FBRR) (Fig. 5B, D and F). In the case of $FeCl_3$, the phloem cells formed with the schizogenous ducts a bluish black ring separating the cortex from the pith.

4. Discussion

The epidermal features seen in stems and leaflets of *S. lorentzii* and

S. marginata reflect a strong adaptation of these trees to environments of high intensity of light and temperature, and low water availability (Arambarri et al., 2011; Roth, 1984; Stace, 1965). The mentioned environmental conditions prevail in the Gran Chaco region where these trees evolved. The high density of non-glandular trichomes on leaflets and stems suggests that they exert a defense role as mechanical barrier against insect herbivores and/or by generating adverse microenvironmental conditions for growth of phytopathogenic microorganisms (War et al., 2012). Regarding glandular trichomes, several plants store hydrophobic defense substances into these structures located at high densities on the surface of aerial parts (Mercado et al., 2014). This is not the case for *Schinopsis* trees which showed a very low density of glandular trichomes in their mature aerial parts together with an important number of uniformly distributed and well developed phloematic schizogenous ducts. Similar characteristics were reported for other Anacardiaceae species of the Rhoeae and Mangiferae tribes (Sant'Anna-Santos et al., 2006; Silva et al., 2000; Carmello et al., 1995). Hence, the chemical defense of full developed leaflets and stems likely relies on compounds secreted to the schizogenous ducts more than on molecules exudated from glandular trichomes. Regarding the TLC analysis, the phenolic compounds of the extracts formed bluish black chelates with $FeCl_3$. Polyphenols bearing in their structures free hydroxyl groups located in a catechol pattern (heptadecylcatechol, catechol, gallic acid and quercetin) and/or a ketone flanked with a hydroxyl group (quercetin and kaempferol) also gave this colour. Phenolics showing only one free hydroxyl group (ferulic, p-coumaric and vanillic acids) coloured orange while the chelating reaction was hindered with polyphenols bearing free hydroxyl groups in a resorcinol pattern (orcinol, olivetol). In the case of the diazonium salts, FBB and FBRR did not reveal the same constituents in the extracts and also showed a different ability to stain the individual polyphenolic species assayed on TLC. This situation was not clearly associated to the pH of the reaction medium. At an acid pH, diazonium salts should primarily couple at the *para* position of the benzene ring and at the *ortho* position when the former one is already occupied. These differences in availability of the *para* and *ortho* positions should be minimal at the mild alkaline pH of FBRR and should become striking at the acidic pH of FBB (Medina, 2011; Harris et al., 1982). Nevertheless, FBRR weakly coloured polyphenols with benzene ring unsubstituted at both *para* and *ortho* positions while it was unable to couple with most of those compounds where *ortho* positions were the only available. In contrast, the acidic FBB coupled with all individual polyphenolic species not visualized with FBRR. The behavioral bias between the expected and the observed azo dye products probably is due to a different coupling reactivity of the Fast Blue RR $ZnCl_2$ and Fast Blue B ½$ZnCl_2$ salts which have one and two azo cations in their molecules, respectively (Sampietro et al., 2013a,b). Irrespective of that, both diazonium salts clearly provided a more polychromatic pattern of polyphenols than $FeCl_3$. The formers stained few constituents of the extracts with the same colour as that observed for the urushiol fraction while the later always gave a uniform and non-discriminating bluish black colour. This situation can be extrapolated to the histochemical tests where $FeCl_3$ was unable to distinguish phenolic lipids from other phenolic compounds in the cross sections of stems and leaflets. FBRR stained the lumen of the schizogenous ducts and their sorrounding epithelial cells with the same colour previously observed for the urushiol fraction in the TLC tests. FBB provided a different colour which indicates that it likely coupled not only to phenolic lipids but also to other phenolic compounds confined in the schizogenous ducts and the epithelial cells. Urushiols were not stained by the diazonium salts in the glandular trichomes or the cuticular layers of leaflets and stems. This fact suggests that the urushiols are available only after the breakdown of leaflets and stems of the *Schinopsis* trees. It is also interesting to note that FBB and $FeCl_3$ stained a high amount of phenolic compounds, other than the urushiols, located at the most adaxial cell layer of the mesophyll in the leaflets and at the outer layer of the photosynthetic parenchymatic cells of the stems. These cells are the most exposed to the solar radiation in these aerial organs. Hence, they might accumulate phenolic compounds to protect themselves against the ultraviolet and infrared radiations.

The histochemical and anatomical analysis of the leaflets and stems of the *Schinopsis* trees indicated that the urushiols are located in the cavities of the phloematic schizogenous ducts and their peripheral epithelial cells. The last ones seem to accumulate and secrete these compounds to the ducts. The urushiols lipids are absent in surface structural features of the organs investigated. A breakdown of the aerial parts is necessary for the release of these compounds. Then, intact organs should be not a threat to humans or animals. FBRR was the most selective reagent for the *in situ* location of the phenolic lipids, FBB allowed to discriminate the presence of other phenolics and $FeCl_3$ had the lowest performance as histochemical reagent.

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