A Staining Protocol for Identifying Secondary Compounds in Myrtaceae

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PROTOCOL NOTE

A STAINING PROTOCOL FOR IDENTIFYING SECONDARY COMPOUNDS IN MYRTACEAE[1]

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- *Premise of the study:* Here we propose a staining protocol using toluidine blue (TBO) and ruthenium red to reliably identify secondary compounds in the leaves of some species of Myrtaceae.
- *Methods and Results:* Leaves of 10 species representing 10 different genera of Myrtaceae were processed and stained using five different combinations of ruthenium red and TBO. Optimal staining conditions were determined as 1 min of ruthenium red (0.05% aqueous) and 45 s of TBO (0.1% aqueous). Secondary compounds clearly identified under this treatment include mucilage in the mesophyll, polyphenols in the cuticle, lignin in fibers and xylem, tannins and carboxylated polysaccharides in the epidermis, and pectic substances in the primary cell walls.
- *Conclusions:* Potential applications of this protocol include systematic, phytochemical, and ecological investigations in Myrtaceae. It might be applicable to other plant families rich in secondary compounds and could be used as a preliminary screening method for extraction of these elements.

Key words: anatomy; Myrtaceae; ruthenium red; staining; toluidine blue.

Ammoniated ruthenium oxychloride (ruthenium red) and the thiazine metachromatic stain toluidine blue (TBO) are regarded as two effective biological stains (Chaffey et al., 2002). Both reagents are often used in plant staining protocols involving hydration, staining, and dehydration (Johansen, 1940; Ruzin, 1999). TBO has been widely used in plant histology to highlight diverse anatomical components including lignified and nonlignified cell walls, nuclei, polyphenols, tannins, and suberin (O'Brien et al., 1964; Crews et al., 2003; Perez-de-Luque et al., 2006). TBO is a cationic stain that binds to tissue anions and gives two main spectra of reaction, purple-pink and green-blue (Baker, 1966). Ruthenium red is a polycationic stain that has applications for electron microscopy (Luft, 1971; Colombo and Rascio, 1977). However, it also has important applications for light microscopy to stain aldehyde-fixed mucopolysaccharides, calcium-dependent reactions, and specific staining of pectic substances, mucilage, and gums (Colombo and Rascio, 1977; Dierichs, 1979; Perez-de-Luque et al., 2006). Improving the visual contrast of these reactions depends upon staining time, concentration, and particular characteristics of the tissue, which might be species dependent (Ruzin, 1999; Zhao et al., 2005). The visualization of these features can be optimized using efficient staining protocols that stain certain chemical compounds contained for these structures (Johansen, 1940; Cutler et al., 2008).

In the case of the family Myrtaceae, compounds such as mucilage, pectins, and polyphenols are abundant in the leaf mesophyll (Wilson, 2011). Mucilage, pectins, and other chemical secretions are regarded as taxonomically significant characters for the family (Schmid, 1980; Keating, 1984; da Silva et al., 2012). Although Myrtaceae is a large family of plants with ca. 5500 species (Wilson, 2011), anatomical studies of leaves are scarce and reports on secondary compounds are limited. A number of species in the family are rich in chemical compounds with medicinal and biochemical activity (Wollenweber et al., 2000; Kytridis and Manetas, 2006). Nevertheless, pharmacological studies rely greatly on plant anatomy, and more staining alternatives are needed in Myrtaceae.

Staining protocols used in Myrtaceae to date mainly involve Safranin O or some combination of Safranin O with Alcian blue, Astra blue, or Fast green (Schmid, 1980; Cardoso et al., 2009; Gomes et al., 2009; Soh and Parnell, 2011). There are a variety of studies regarding the staining of plant tissues with ruthenium red and TBO (Littlefield and Wilcoxson, 1962; Leiser, 1968; Western et al., 2001; Stpiczyńska and Davies, 2009). However, there are no published studies about optimization of staining procedures for a specific plant family or taxonomic group. Due to the presence of particular chemicals in the species of the family, an alternative staining protocol may improve the resolution of tissues in anatomical sections. Here we report

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TABLE 1. Staining treatments applied in this study based on duration of staining.

Treatment	Duration of staining (min)	
	Ruthenium red (0.05% w/v)	TBO (0.1% w/v)
T1	2	1
T2	2	0.5
T3	0	2
T4	2	0
T5	1	0.75

an experiment using different combinations and duration of staining with ruthenium red and TBO, so as to reliably identify secondary compounds in the family Myrtaceae.

METHODS AND RESULTS

***Sampling, fixation, and sectioning*—**Leaves of Australian and South American Myrtaceae from different genera were collected from the natural habitat of the species. Species were selected from different genera to encompass a diversity of leaf structures. Details of taxa, location, and collector numbers are provided in Appendix 1. Voucher specimens are currently housed at Queensland Herbarium (BRI), Brisbane, Australia, and will be deposited at Forestry Sciences Herbarium (EIF), Universidad de Chile, Santiago, Chile, and the Museo Nacional de Historia Natural (SGO), Santiago, Chile, at a later date. Leaves were fixed in formalin–acetic acid–alcohol (FAA) for 24–48 h depending upon whether the species had soft or hard leaves. Composition of FAA (for 100 mL) was 90 mL of 50% ethanol, 5 mL of glacial acetic acid, and 5 mL of formalin 37–40% (Johansen, 1940). Fixed material was dehydrated through a graded ethanol series and embedded in paraffin wax (Johansen, 1940; Ruzin, 1999). Transverse sections (5 µm thickness) were cut using a Leica RM2245 rotary microtome (Leica Biosystems, Buffalo Grove, Illinois, USA).

***Staining procedure*—**Samples were deparaffinized with xylene, and then gradually hydrated through a decreasing alcoholic series (ethanol 100%, 90%, 70%, 50%, distilled water). Histochemical staining of sections was performed using a 0.1% (w/v) solution of TBO (Amresco, Solon, Ohio, USA) in distilled water and 0.05% (w/v) of ruthenium red (Sigma-Aldrich Co., St. Louis, Missouri, USA) in distilled water following Jensen (1962). Samples were stained with one or both reagents for different periods of time according to five treatments, namely, T1, T2, T3, T4, and T5 (Table 1). All specimens were subjected to these treatments (T1–T5) to determine optimal staining conditions that can be used to reliably identify anatomical characters across Myrtaceae. After staining, slides were dehydrated using an increasing ethanol series (50%, 70%, 90%, 100%, xylene) and mounted with DPX (Sigma-Aldrich Co.). The sections were observed using a Nikon SMZ-800 Stereoscopic light microscope (Nikon Eclipse 50i compound), and pictures were taken using the NIS Elements digital image analysis software (Nikon Instruments, Amsterdam, The Netherlands). Interpretation of colors from histochemical staining was based on O'Brien et al. (1964), Chaffey et al. (2002), Zhao et al. (2005), and Perez-de-Luque et al. (2006).

A total of 10 sections were stained per treatment of each species, which corresponds to ca. 500 sections. The entire staining experiment, from deparaffinization to mounting, takes approximately two hours. Details of the staining protocol and cautionary comments are presented in Appendix 2.

***Optimal staining protocol*—**Histochemical reactions in leaves were notably different depending upon treatment. Staining with ruthenium red for 1 min and counterstaining with TBO for 45 s (T5) proved to be the most effective combination for differentiating secondary compounds based on color (Table 2). T5 also proved to be the most consistent treatment of the experiment, staining secondary compounds with similar colors and contrast uniformly in all the species. Under this treatment, polyphenols, carboxylated polysaccharides, mucilage, and pectins were clearly visible in different parts of the leaf (Fig. 1). Treatment T5 allowed a proper contrast between the cuticle (blue-green for polyphenolic compounds) and the epidermal cells of most of the species. Apparently, *Myrceugenia parvifolia* (DC.) Kausel and *Luma apiculata* (DC.) Burret do not present polyphenols in the cuticle, as both species lack blue staining in this structure (Fig. 1E, 1G). Vascular bundles also presented better-defined elements using this treatment, showing clear differentiation between lignified secondary cell walls and nonlignified primary walls. Lignified vessels and fibers were stained blue-green with TBO, allowing excellent visual contrast. On the other hand, nonlignified primary cell walls in the xylem, secondary phloem, and nonvascular tissues were stained red with ruthenium red, a result similar to that seen in other studies (Zhao et al., 2005; Perez-de-Luque et al., 2006). Positive staining with ruthenium red was suitable for observing pectic substances in the middle lamella of nonlignified primary cell walls. Ruthenium red also allowed direct observation of mucilage in the mesophyll of most of the species.

Even though T5 had similar results through all the species in terms of secondary compounds, there are some taxa with special anatomical features that stained differently. The palisade parenchyma cells of *Gossia floribunda* (A. J. Scott) N. Snow & Guymer and *Eugenia reinwardtiana* (Blume) DC. were stained strongly and appear darker than those of other species (Fig. 1), indicating the presence of tannins and polysaccharides. The epidermal cells of some species (*G. floribunda*, *E. reinwardtiana*, *Ugni molinae* Turcz.) contain tannins (stained blue) and carboxylated polysaccharides (stained pink), while the epidermal cells of other species (*M. parvifolia*, *Syzygium australe* (J. C. Wendl. ex Link) B. Hyland, *Waterhousea floribunda* (F. Muell.) B. Hyland) lack these compounds. The phloem sieve tube members of the midrib in certain taxa (such as *Acmena smithii* (Poir.) Merr. & L. M. Perry) have a dark-staining content, potentially tannins, while *M. parvifolia*, *U. molinae*, and *W. floribunda* give contrasting examples of taxa without tannins in the phloem.

The species *L. apiculata* and *M. parvifolia* reacted somewhat differently to treatment T5, showing a different pattern and intensity of colors. Histochemical staining revealed an abundance of pectic substances and mucilage in the mesophyll of *L. apiculata*, with a predominance of red staining over blue compared to the other species when treated with T5. In the case of *M. parvifolia*, staining was slightly weaker than the other nine species; however, anatomical elements and secondary compounds were clearly differentiated.

TABLE 2. Secondary compounds (and colors) identified with each treatment (T1–T5) of all the species based on the color resulting from the staining process.

Taxon	Mucilage (red-pink)					Polyphenols, tannins, lignins (blue-green)					Carboxylated polysaccharides (pink)					Pectic substances and some tannins (red)				
	T1	T2	T3	T4	T5	T1	T2	T3	T4	T5	T1	T2	T3	T4	T5	T1	T2	T3	T4	T5
Acmena smithii	+	+	–	+	+	+	+	–	–	+	+	–	–	–	+	–	+	–	+	+
Gossia floribunda	+	+	–	+	+	+	–	–	–	+	+	+	–	+	+	+	+	–	+	+
Decaspermum humile	–	+	–	+	+	–	–	–	–	+	+	+	–	+	+	+	+	–	+	+
Eugenia reinwardtiana	–	+	–	+	+	–	–	–	–	+	+	+	–	+	+	–	+	–	+	+
Luma apiculata	+	+	–	+	+	+	–	–	–	+	+	–	–	–	+	+	+	–	+	+
Myrceugenia parvifolia	–	+	–	+	+	+	–	–	–	+	+	+	–	–	+	–	+	–	+	+
Myrteola nummularia	–	+	–	+	+	+	–	–	–	+	+	+	–	–	+	–	+	–	+	+
Syzygium australe	–	+	–	+	+	+	+	–	–	+	+	+	–	–	+	–	+	–	+	+
Ugni molinae	+	+	–	+	+	+	+	–	–	+	+	–	–	+	+	–	+	–	+	+
Waterhousea floribunda	+	+	–	+	+	+	+	–	–	+	+	–	–	–	+	–	+	–	+	+

Note: + = positive staining of secondary compounds; – = no staining of secondary compounds.

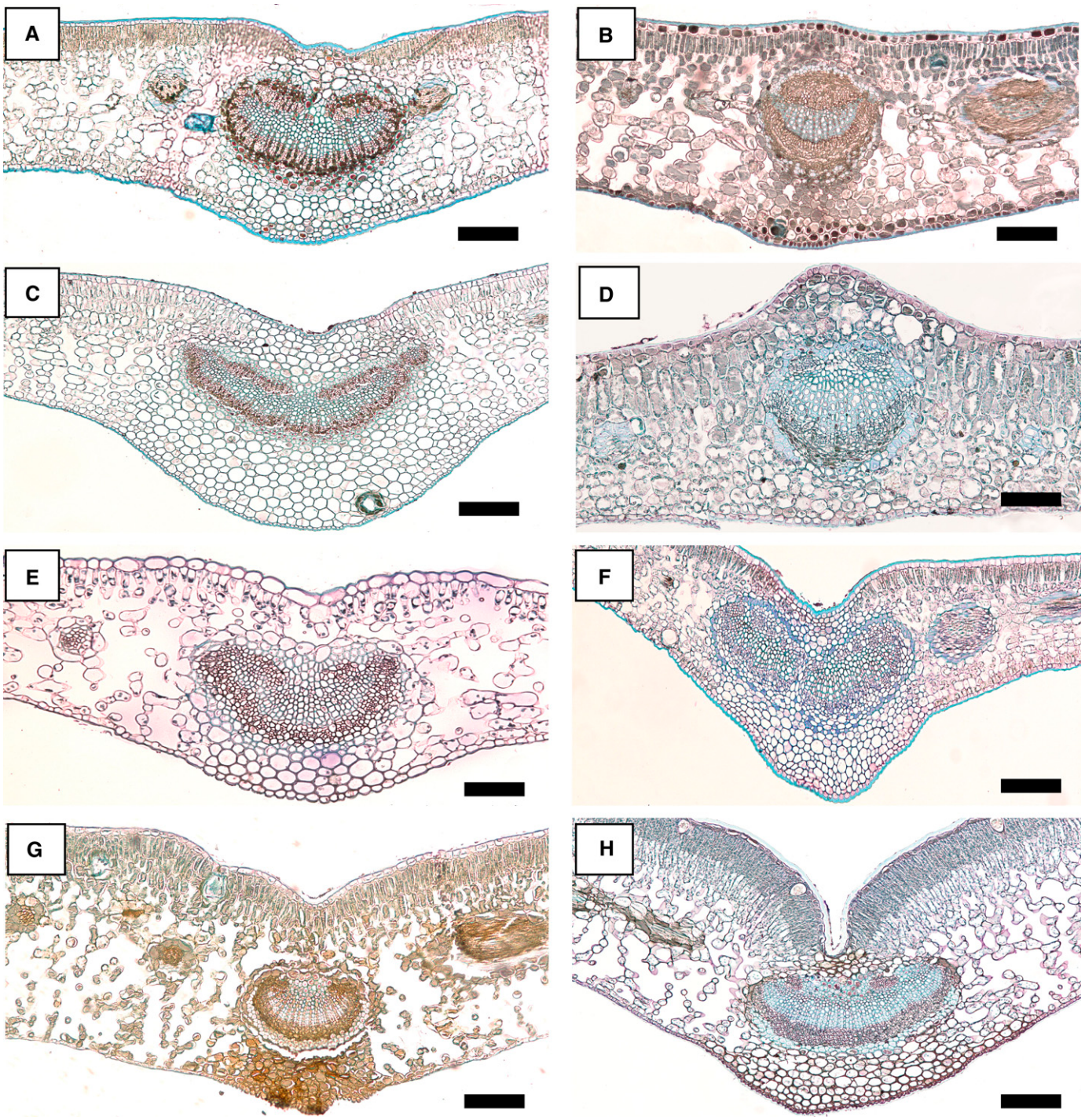

Fig. 1. Transverse light micrographs (LM) of leaves of some species of Myrtaceae stained with the staining treatment T5. (A) *Acmena smithii*: Clear highlight of lignified cells (blue) and mucilage (red). (B) *Eugenia reinwardtiana*: Carboxylated polysaccharides in epidermis. (C) *Syzygium australe*: Phloem cells stained red and xylem stained blue-green. (D) *Gossia floribunda*: Mesophyll cells containing dark stained pigments, probably tannins. (E) *Myrceugenia parvifolia*: Pectic substances in primary cell walls observed in spongy parenchyma (red). (F) *Waterhousea floribunda*: Cuticle with polyphenols stained blue and fibers with lignin stained blue-purple. (G) *Luma apiculata*: Red staining of pectic substances and mucilage. (H) *Ugni molinae*: Mucilage in spongy parenchyma and clear difference between polyphenols in xylem (lignin) and phloem. Scale bars = 100 µm.

***Comparison between treatments*—**Treatment T1 showed similar results to T5 in terms of staining reaction, but some secondary compounds are not clearly visible with T1. Polyphenols (e.g., lignin) are stained red in treatments T1, T2, and T4, without clear differentiation between the midrib fibers and the mesophyll cells due to weak reaction of TBO (Fig. 2). Similarly, the xylem and phloem are not easily differentiable under treatments T2, T3, and T4 (Fig. 3). On the other hand, treatment T5 allowed the clear observation of lignin in fibers stained blue for this dye (Fig. 2) and polyphenols in the cuticle (Fig. 3). Although there are

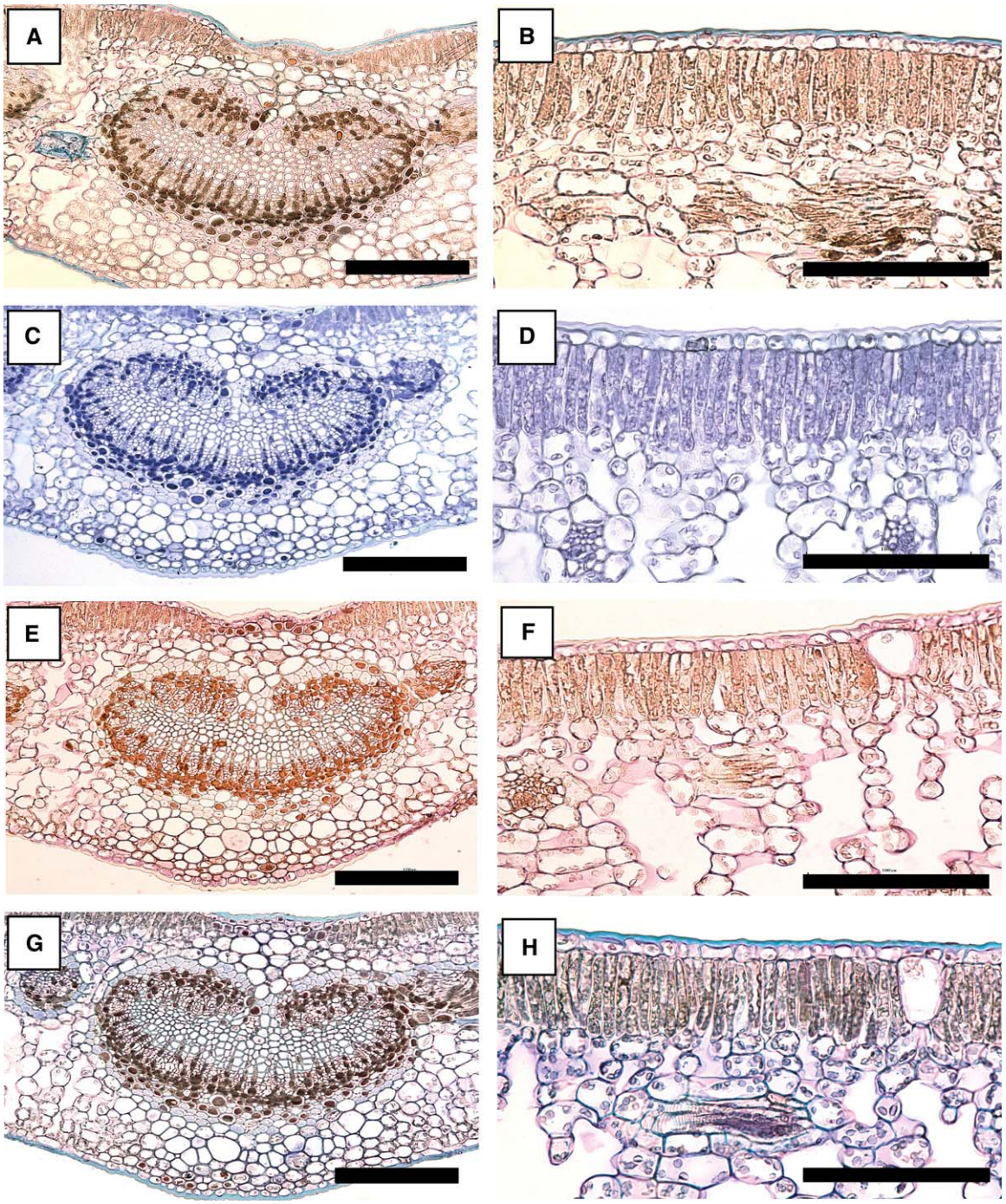

Fig. 2. Transverse light micrographs (LM) of leaves of *Acmena smithii*, showing comparisons between treatments T2 (A, B), T3 (C, D), T4 (E, F), and T5 (G, H). Treatment T1 showed similar results to T5. (A, B) Polyphenols highlighted in the cuticle (blue) and mucilage (red); however, the lignified elements in the midrib are not differentiated. (C, D) Secondary compounds are not differentiated for masking of TBO. (E, F) Secondary compounds are not differentiated for masking of ruthenium red. (G, H) Secondary compounds are clearly visible with treatment T5, e.g., polyphenols in the cuticle, xylem, and fibers (lignin, blue); mucilage and pectic substances in the mesophyll (red); tannins in the phloem (red); and carboxylated polysaccharides in the epidermis (pink). Scale bars = 100 μm.

some differences if the lignin is fresh (in situ), polychromatic staining with TBO is a reliable method to identify this compound when the staining time is optimal (O'Brien et al., 1964). Overstaining with ruthenium red (T2, T4) produced homogeneous red staining through the samples without any colored enhancement of secondary compounds except for mucilage and pectic substances (Figs. 2, 3). In the case of treatment T4, the action of TBO might be neutralized by ruthenium red, which is regarded as a stronger stain (Dierichs, 1979; Chaffey et al., 2002). Treatment T3 with TBO for 2 min resulted in blue overstaining without the optimal polychromatic reaction in tissues.

The combination that showed the best results (T5) might be proposed as an alternative protocol to existing ones involving different stains in Myrtaceae. Procedures involving Safranin O or a combination of Safranin O with Alcian

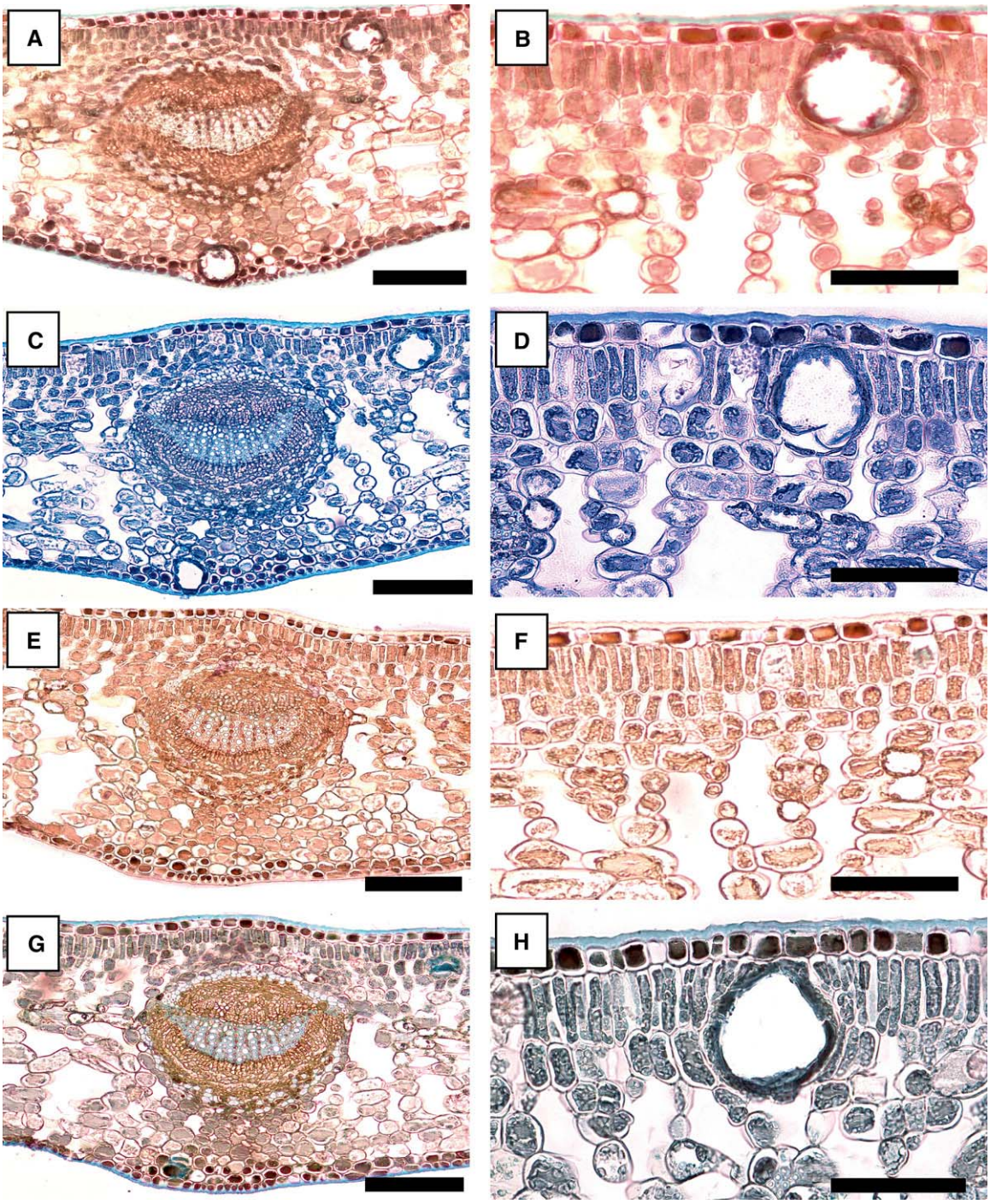

Fig. 3. Transverse light micrographs (LM) of leaves of *Eugenia reinwardtiana*, showing comparisons between treatments T2 (A, B), T3 (C, D), T4 (E, F), and T5 (G, H). Treatment T1 showed similar results to T5. (A, B) Only polyphenols in the cuticle (blue) are differentiated as the red staining is predominant. (C, D) Secondary compounds are not differentiated for masking of TBO, except for the cuticle. (E, F) Secondary compounds are not differentiated for masking of ruthenium red. (G, H) Secondary compounds are clearly visible in treatment T5, and the xylem and phloem are clearly differentiated, e.g., polyphenols in the cuticle, xylem, and fibers (lignin, blue); mucilage and pectic substances in the mesophyll (red); tannins in the phloem (red); and carboxylated polysaccharides in the epidermis (pink). Red tannins in the epidermis are observable with all the treatments. Scale bars = 100 μm.

blue, Astra blue, or Fast green have shown satisfactory results in anatomical studies on the family. However, there are no published studies supporting the use of these stains and their advantages. In addition to the quality of the enhancement of secondary compounds, the use of ruthenium red and TBO has advantages in terms of time and safety. Safranin O is a regressive stain and needs between 2 and 24 h to be effective; furthermore, it requires destaining in distilled water (Johansen, 1940; Ruzin, 1999). Safranin O also requires differentiation with picric acid, hydrochloric acid, or tannic acid, which are regarded as unstable reagents (Ashbrook and Houts, 2003). On the other hand, the use of ruthenium red and TBO does not require much time (2–3 min) or dangerous reagents.

CONCLUSIONS

In this paper, we introduced a double staining protocol using ruthenium red and TBO. We have evaluated a number of different staining treatments with these reagents to reliably differentiate secondary compounds in leaves of some species of Myrtaceae. The best combination was determined as 1 min of ruthenium red and 45 s of TBO (treatment T5). Under this treatment, a number of secondary leaf compounds can be clearly identified: polyphenols, mucilage, carboxylated polysaccharides, and pectic substances. This procedure enhances the contrast of secondary compounds, which are visible in a wide range of colors (green-blue-red-pink). The applicability, safety, and effectiveness are the main advantages of this protocol when compared to similar staining procedures used in the family. Other staining protocols used in Myrtaceae require more time and involve unstable reagents, such as hydrochloric acid or tannic acid, or even explosive compounds such as picric acid. This protocol involves relatively few reagents and offers the option of adjusting and varying the duration at each stage of the process. This procedure might be an alternative to commonly used staining protocols in Myrtaceae. Although the best results were obtained using a particular combination of stains, it is advisable to test the full procedure to detect differences in the results with other taxonomic groups. Identification of secondary compounds in leaves of Myrtaceae is highly important for systematic, phytochemical, and ecological studies. This protocol could be used as a screening method for deeper study or extraction of these compounds.

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Appendix 1. Taxa, vouchers, geographic locality, and GPS coordinates of samples collected for this study.

Taxon	Collection no.[a]	Collection locality	Geographic coordinates
Acmena smithii (Poir.) Merr. & L. M. Perry	Reta-032.1–Reta-032.2	Robina, QLD, Australia	28°4′46″S, 153°23′17″E
Gossia floribunda (A. J. Scott) N. Snow & Guymer	Reta-031.1–Reta-031.2	Cooroibah, QLD, Australia	26°21′44″S, 152°59′3″E
Decaspermum humile (Sweet ex G. Don) A. J. Scott	Reta-029.1–Reta-029.2	Enoggera, QLD, Australia	27°25′21″S, 152°59′26″E
Eugenia reinwardtiana (Blume) DC.	Reta-028.1–Reta-028.2	Robina, QLD, Australia	28°4′46″S, 153°23′17″E
Luma apiculata (DC.) Burret	Reta-026.1–Reta-026.2	Osorno, Chile	40°34′0″S, 73°9′0″W
Myrceugenia parvifolia (DC.) Kausel	Reta-021.1–Reta-021.2	Puerto Montt, Chile	41°28′18″S, 72°56′12″W
Myrteola nummularia (Poir.) O. Berg	Reta-03.1–Reta-03.2	Futrono, Chile	40°7′28″S, 72°22′51″W
Syzygium australe (J. C. Wendl. ex Link) B. Hyland	Reta-034.1–Reta-034.2	Kawana Island, QLD, Australia	26°43′11″S, 153°7′41″E
Ugni molinae Turcz.	Reta-04.1–Reta-04.2	Talcahuano, Chile	36°43′0″S, 73°7′0″W
Waterhousea floribunda (F. Muell.) B. Hyland	Reta-030.1–Reta-030.2	Enoggera, QLD, Australia	27°25′21″S, 152°59′26″E

Note: QLD = Queensland.

[a] Housed at BRI (Queensland Herbarium, Brisbane, Australia). Duplicates will be deposited at SGO (Museo Nacional de Historia Natural, Santiago, Chile) and EIF (Forestry Sciences Herbarium, Universidad de Chile, Santiago, Chile) during 2014.

Appendix 2. Description of the protocol and troubleshooting. CAS = Chemical Abstracts Service.

Chemicals (vendors) used in this study:

Xylene (Ajax Finechem Pty, Taren Point, New South Wales, Australia). CAS registry no.: 1330-20-7

Ethanol (Chem-Supply Pty, Gillman, South Australia, Australia). CAS registry no.: 64-17-5

Ruthenium red powder (Sigma-Aldrich Co., St. Louis, Missouri, USA). CAS registry no.: 11103-72-3

Toluidine blue powder (TBO) (Amresco, Solon, Ohio, USA). CAS registry no.: 92-31-9

DPX (Sigma-Aldrich Co., St. Louis, Missouri, USA). CAS registry no.: 14208-10-7

Distilled water

Equipment:

Glass staining dishes with glass lids

Slide racks

Trays for drying slides

Paper towels

Fume hood

Nitri-Solve flock-lined gloves (Showa Best Glove, Menlo, Georgia, USA) are recommended when handling xylene, and nitrile gloves for all other reagents.

Safety goggles

Microscope slides

Cover slips (24 × 24 mm) and long cover slips (24 × 50 mm)

Dropper bottle

Forceps, spoon, spatula

Preparation of staining solutions:

Ruthenium red (Jensen, 1962)

1. Dissolve 0.3 g of ruthenium red powder in 100 mL of distilled water

2. Stir for 15–20 min, without heat, until completely dissolved

3. Filter after stirring

4. Store in Schott bottle (Schott North America, Elmsford, New York, USA), refrigerated to prevent growth of microorganisms

Freshly prepared solution should look black in color.

Toluidine blue

1. Dissolve 0.1 g of toluidine blue in 100 mL of distilled water

2. Stir gently

3. Filter

4. Store in Schott bottle, refrigerated to prevent growth of microorganisms

Histochemical staining procedure protocol:

1. Place 20 staining dishes in a fume hood. Fill the dishes with the following solutions: 2× ethanol 50%, 2× ethanol 70%, 2× ethanol 90%, 2× ethanol 100%, 4× xylene, 1× xylene : ethanol (1 : 3), 5× distilled water, 1× aqueous solution of ruthenium red, and 1× aqueous solution of toluidine blue.

2. Place slides with paraffin sections in slide racks and transfer to different solutions and stains using the sequence and duration outlined below:

Step	Solution	Purpose	Duration (estimated intervals)
1–2	Xylene	Deparaffinization	15 min (2 changes)
3	Xylene : ethanol	Hydration	10 min
4	Ethanol 100%	Hydration	5 min
5	Ethanol 90%	Hydration	5 min
6	Ethanol 70%	Hydration	5 min
7	Ethanol 50%	Hydration	5 min
8	Distilled water	Hydration	5 min
9	Ruthenium red	Staining	Variable
10	Distilled water	Removal of excess stain	30 s
11	Distilled water	Removal of excess stain	30 s
12	Toluidine blue	Counterstaining	Variable
13	Distilled water	Removal of excess stain	30 s
14	Distilled water	Removal of excess stain	30 s
15	Ethanol 50%	Dehydration	5 min
16	Ethanol 70%	Dehydration	5 min
17	Ethanol 90%	Dehydration	5 min
18	Ethanol 100%	Dehydration	5 min
19–20	Xylene	Drying	10 min (2 changes)

3. Check staining result under a microscope after step 14 before proceeding with dehydration. Adjust staining time if needed.

4. Mount and apply cover slip with DPX (or Entellan) mounting medium.

5. Lay cover slips out on a sheet of blotting paper and wipe clean to remove dust. Place an elongated drop of mountant (DPX) across the middle of each cover slip. Remove a slide from the xylene (Step 20 above), drain off the excess liquid and lay it, section side down, over the cover slip. Repeat for each slide, making sure that air bubbles are not trapped beneath the cover slip. If bubbles are present, press them gently toward the edge of the cover slip with a mounted needle. Wipe around the slide with a tissue to remove excess xylene or mountant. Dry the slides on a flat surface in the fume hood until the mountant has hardened sufficiently to allow handling.