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Small and hard seeds: a practical and inexpensive method to improve embedding techniques for light microscopy

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ABSTRACT

The traditional techniques employed for embedding plant samples are not suitable for the proper processing of small seeds with hard seed coats. Usually, these seeds are broken off from blocks during microtomy, which limits the technical success of this procedure. In this study, Melastomataceae seeds of 101 species were treated prior to embedding in historesin according to three treatments: (1) control, using the standard procedure; (2) fixation and subsequent softening with Franklin solution, glycerin and heating in a water bath; and (3) softening with Franklin solution and subsequent fixation (glycerin and heating in a water bath were also included). For Melastomataceae species, the second treatment provides the best results, and we were able to produce very good sections of the entire seed. Small hard seeds, similar to those found in Melastomataceae, are better embedded when they are softened after subjected to fixation. A combination of softening techniques is necessary to improve the embedding process and to obtain high-quality sections of the embedded samples. In this study, we established a practical, slightly toxic and inexpensive methodology to ensure good preparations for light microscopy that can be applied to a wide range of subjects related to seed science.

Key words: Anatomy, historesin embedding, Melastomataceae, softening

Introduction

The importance of seeds to the reproductive success of Angiosperms is unquestionable. Data on the structure of the embryo are very important for understanding processes such as dormancy, germination (Goebel 1898) and frugivory (Silveira *et al.* 2013) and to recognize phylogenetic relationships in specific families and genera (Martin 1946; Corner 1976; Martin & Michelangeli 2009). However, the embryo morphology of some species cannot be described without microscopic preparation because the small seed size and/or the hardness of the seed coat hinder this process. Furthermore, many species with small and hard seeds demonstrate no developed embryo even in maturity, although they appear to be morphologically similar to embryonic seeds; this has been described for *Cytinus* (Vega & Carmo-Oliveira 2007) and was also observed by our group in some Melastomataceae. Thus, the lack of information regarding the presence or absence of the embryo, particularly in small seeds, can result in erroneous inferences regarding dormancy and germination. Moreover, many features of the seed or even the embryo are useful for taxonomic and phylogenetic purposes (Martin 1946; Corner 1976). However, detailed studies on the anatomy of small and hard seeds are scarce because these features constitute complex technical barriers to anatomical analyses.

Among the families with this type of seed, which lack of structural data on their reproductive structures, Cytinaceae, Apodanthaceae, Mistratemonaceae, Hydnoraceae, Rafflesiaceae (Vega & Carmo-Oliveira 2007), and Melastomataceae stand out. This encouraged our studies with Melastomataceae. The small size, the rigidity of the seed coat and its consequent impermeability tend to limit the use of conventional anatomical techniques. Seeds with these traits are not properly embedded in historesin (Vega & Carmo-Oliveira 2007), paraffin or paraplast (personal observations). Under these conditions, it is common that sections of embedded samples are not adequate for light microscopy analyses, and the embryo is rarely preserved.

According to Johansen (1940), the seed coat hardness necessitates the use of processes similar to those applied to woody tissues. Werker (1997) showed that seed coat hardness may be due to the presence of lignified layers (e.g., sclereids) or the deposition of phenolic compounds, both of which are commonly found in the seeds of several families.

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In many seed science research fields, high-quality anatomical preparations of seeds are essential. Several researchers aimed to obtain good preparations with small (Vega & Carmo-Oliveira 2007) and hard seeds (Silveira *et al.* 2012; 2013), but complete success was not achieved until recently.

Considering these findings, the aim of this study was to develop an effective and inexpensive methodology for the processing of small and hard seeds in order to ensure proper anatomical analyses.

Material and methods

Plant material and fixation

Mature seeds from 101 species of Melastomataceae (Table 1) were collected from ripe and dry fruits or dried specimens from the herbaria BHCB, CAS, FLAS, HUFU, NY and UCPB. As a model to illustrate these results, we selected *T. laniflora*, a species whose seeds reflect all technical barriers encountered in the evaluated species.

All the seeds were fixed in formalin-aceto-50% ethanol (Johansen 1940) for 48 hours according to the treatments described below. To ensure that the entire surface of the seeds was immersed in the fixative, all of the samples were subjected to rotation (Eppendorf Centrifuge 5415D) at 10 rpm for 2 minutes and maintained under vacuum for 24 hours thereafter. Three treatments were performed: the standard embedding methods (treatment 1) and two alternative softening techniques (treatment 2 and 3).

Treatment 1 (T1) – Control, standard procedure

We used the more conventional treatment for embedding: the seeds were fixed, dehydrated in an ethanol series (50, 60, 70, 80, 90 and 95%), and subsequently embedded in (2-hydroxyethyl)-methacrylate (HEMA, Leica™ Historesin Embedding Kit). To achieve this, the samples were kept in pre-infiltration solution (1:1, 95% ethanol: infiltration solution, which was prepared with 50 mL of liquid basic resin plus 0.5 g of activator) for 12-18 hours (overnight) and then remained in the infiltration solution for one week in a refrigerator (± 5°C). After infiltration, the samples were embedded (embedding medium: 15 mL of infiltration solution plus 1 mL of hardener) and kept for an additional week in the freezer (± -18°C) to retard polymerization (Paiva *et al.* 2011). The times for infiltration and embedding were extended because the greatest difficulties in processing these types of samples are commonly found in the infiltration and embedding steps. After a week of embedding, the histomolds with samples were kept at room temperature for polymerization.

When the blocks were polymerized, 6-µm-thick longitudinal sections were obtained using disposable steel blades and a rotary microtome (Zeiss™ Hyrax M40). The sections were stained with 0.05% toluidine blue at pH 4.7 (O'Brien *et al.* 1964 modified) and mounted with Entellan™.

T1 was performed to determine whether the more conventional resin embedding protocol was adequate for obtaining good microscopic preparations of small and hard seeds.

Treatment 2 (T2) – Fixation and subsequent softening

After fixation, samples of the seeds were softened in the Franklin maceration solution (Franklin 1945) prepared with concentrated glacial acetic acid and 30% hydrogen peroxide (1:1 v/v). These samples were kept in an incubator at 60°C for 4 to 6 hours and then washed with distilled water to completely remove the solution. After this procedure, the seeds were dehydrated in an ethanol series (10, 20, 30, 40, 50, 60, 70%), and each alcohol was mixed with 10% glycerin, and the samples were kept in a water bath at 30°C for 1 hour after each change.

The samples were stored in 70% ethanol + 10% glycerin for three days and then dehydrated in 80, 90, and 95% ethanol. The pre-infiltration, infiltration, embedding, polymerization, sectioning and staining steps were the same as in T1.

This treatment was designed to evaluate the effect of the combined techniques of maceration (Franklin 1945) and softening (using glycerin and water bath) on the samples after fixation.

Treatment 3 (T3) – Softening and subsequent fixation

The seeds were softened in the maceration solution described by Franklin (1945), as in T2, and washed with distilled water to completely remove the solution prior to fixation (Johansen 1940). Because the samples were already in a 50% ethanol solution, the fixative was washed with this alcohol, and the samples were dehydrated in 60 and 70% ethanol, both of which were mixed with 10% glycerin, and kept in a water bath at 30°C for one hour after each change.

As in T2, the samples were stored in 70% ethanol plus 10% glycerin for three days and then subjected to the same subsequent steps.

T3 was performed to verify whether softening prior to fixation would be more effective for improving embedding.

Results and discussion

The selection of the best method requires consideration of the maintenance of seed integrity as a whole. In this study, we determined good conditions for observing the seed coat and embryo. Analyses of the sections obtained using three treatments revealed different results both in *T. laniflora* seeds (Figure 1A-F) and 100 other species of Melastomataceae under investigation (Table 1).

The sections obtained with T1 did not allow for analyses of the embryo (Figure 1A). In the seed coat, the thickened phenolic walls were well preserved, but thin pecto-celllulosic walls (anticlinal and outer periclinal wall) collapsed (Figure 1D).

Table 1. A list of the 101 species of Melastomataceae tested in this study. The species are grouped into tribes according to the literature (Fritsch *et al.* 2004; Goldenberg *et al.* 2008; 2012; Michelangeli 2008), and data on the Herbarium and the collector are provided.

Species	Herbarium	Collector
Melastomeae tribe		
1. *Aciotis polystachya* (Bonpl.) Triana	BHCB	M.J.R. Rocha 823
2. *Acisanthera bivalvis* (Aubl.) Cogn.	BHCB	M.J.R. Rocha 871
3. *Comolia sessilis* Triana	BHCB	F.A.O. Silveira 18
4. *Comolia sertularia* Triana	BHCB	F.A.O. Silveira 19
5. *Macairea lasiophylla* (Benth.) Wurdack	BHCB	M.J.R. Rocha 873
6. *Nepsera aquatica* (Aubl.) Naudin	BHCB	M.J.R. Rocha 821
7. *Tibouchina cardinalis* Cogn.	BHCB	F.A.O. Silveira 20
8. *Tibouchina heteromalla* (D. Don) Cogn.	BHCB	F.A.O. Silveira 21
9. *Siphanthera arenaria* (DC.) Cogn.	HUFU	R.Romero e J.N. Nakajima 6005
10. *Siphanthera dawsonii* Wurdack	HUFU	J.N.Nakajima 1943
Merianieae tribe		
11. *Cambessedesia hilariana* (Kunth) DC.	HUFU	I.M. Franco *et al.* 356
12. *Cambessedesia regnelliana* Cogn.	HUFU	C.M.Rodrigues 20
13. *Dolichoura spiritusanctensis* Brade	UPCB	L. Kollmann 2247
14. *Graffenrieda limbata* Triana	UPCB	R. Goldenberg 998
Miconieae tribe		
15. *Anaectocalyx bracteosa* Triana	CAS	Dorr & Barnett 7563
16. *Calycogonium glabratum* (Sw.) DC.	CAS	Michelangeli et al. 1478
17. *Charianthus alpinus* (Sw.) R.A. Howard	CAS	Wilbur et al. 7788
18. *Charianthus dominicensis* Penneys & Judd	CAS	Wilbur et al. 8080
19. *Clidemia caudata* Wurdack	NY	D. Restrepo & A. matapi 520
20. *Clidemia capitellata* (Bonpl.) D. Don	UPCB	M. Oliveira 861
21. *Clidemia domingensis*	NY	A.H. Liogier 13185
22. *Clidemia garciabarrigae* Cogn.	CAS	Valdespino et al. 596
23. *Clidemia globuliflora* (Cogn.) L.O. Williams	NY	W.C. Burger & R.L. Liesner 6761
24. *Clidemia urceolata* DC.	UPCB	R. Goldenberg 1089
25. *Conostegia lasiopoda* Benth.	CAS	Croat 68155
26. *Conostegia montana* (Sw.) D. Don ex DC.	UPCB	F Almeda 3958
27. *Conostegia oerstediana* O. Berg ex Triana	CAS	Cowan & Murillo 4526
28. *Conostegia xalapensis* (Bonpl.) D. Don	CAS	Miller & Campos 2945
29. *Eriocnema fulva* Naudin	BHCB	E.F. Borsali 154, 156
30. *Leandra aurea* (Cham.) Cogn.	BHCB	F.A.O. Silveira, 22
31. *Leandra dichotoma* (Pav. ex. D. Don) Cogn.	CAS	Knapp et al. 1736
32. *Leandra divaricata* Cogn.	CAS	Clarke 2504
33. *Leandra granatensis* Gleason	CAS	Mendieta 9-34
34. *Leandra melastomoides* Raddi	BHCB	F.A.O. Silveira 23
35. *Leandra mexicana* (Naudin) Cogn.	CAS	Nee et al. 46915
36. *Leandra reversa* (DC.) Cogn.	UPCB	R. Goldenberg 1104
37. *Leandra subulata* Gleason	CAS	McPherson 9687
38. *Maieta poeppigii* Mart. ex. Cogn.	CAS	Henkel & Williams 2167
39. *Mecranium acuminatum* (DC.) Skean	CAS	Judd et al. 2954
40. *Mecranium ovatum* Cogn.	CAS	Judd et al. 5184

Continues.

Table 1. Continuation.

Species	Herbarium	Collector
41. *Miconia aliquantula* Wurdack	CAS	Hahn 3605
42. *Miconia argentea* (Sw.) DC.	CAS	Carballo 1133
43. *Miconia astroplocama* Donn. Sm.	CAS	Brandbyge & Asanza 31987
44. *Miconia bracteolata* (Bonpl.) DC.	NY	W.H. Camp E-4176
45. *Miconia centrodesma* Naudin	UPCB	Amorim 5758
46. *Miconia corymbiformis* Cogn.	CAS	Ortiz et al. 307
47. *Miconia costaricensis* Cogn.	CAS	Hahn 634
48. *Miconia crocata* Almeda	CAS	Almeda et al. 6388
49. *Miconia denticulata* Naudin	CAS	Valencia 2342
50. *Miconia dielsiana* Urb.	NY	W.S. Judd et al. 8217
51. *Miconia dolichorrhyncha* Naudin	CAS	Shepherd 309
52. *Miconia duckei* Cogn.	NY	M. Rimachi 11362
53. *Miconia foveolata* Cogn.	CAS	Howard 16624
54. *Miconia furfuracea* (Vahl) Griseb.	NY	R.L. Wilbur et al. 8191
55. *Miconia globuliflora* Cham. ex Triana	NY	L.M. Andrews 3-64
56. *Miconia goniostigma* Triana	CAS	Sytsma 1513
57. *Miconia gratissima* Benth. ex Triana	CAS	Arroyo et al. 748
58. *Miconia mirabilis* (Aubl.) L.O. Williams	UPCB	R. Goldenberg 1091
59. *Miconia multiplinervia* Cogn.	CAS	Almeda et al. 6163
60. *Miconia nitidissima* Cogn.	NY	F.A. Michelangeli et al. 821
61. *Miconia pachyphylla* Cogn.	NY	E.L. Little, Jr. 21621
62. *Miconia paradoxa* (Mart. ex DC.) Triana	UPCB	V.C. Souza 22163
63. *Miconia prasina* (Sw.) DC.	CAS	Davidse et al. 35603
64. *Miconia procumbens* (Gleason) Wurdack	CAS	Hammel et al. 16388
65. *Miconia rubiginosa* (Bonpl.) DC.	UPCB	F. Almeda 9531
66. *Miconia schlimii* Triana	CAS	Lundell & Contreras 19881
67. *Miconia sellowiana* Naudin	UPCB	R. Goldenberg 732
68. *Miconia sessilifolia* Naudin	CAS	Kessler et al. 4255
69. *Miconia septentrionalis* Judd & R.S. Beaman	FLAS	Jiménez & Polanco 1853
70. *Miconia stenostachya* DC.	UPCB	F. Almeda 8929
71. *Miconia tomentosa* (Rich.) D. Don ex DC.	UPCB	C. Fernando-Bulhão 537
72. *Miconia triplinervis* Ruiz & Pav.	CAS	Contreras 7899
73. *Necranium gigantophyllum* Britton	NY	W.G.F. et al. 11953
74. *Ossaea brenesii* Standl.	CAS	Hamilton & Davidse 2661
75. *Ossaea capillaris* (D. Don) Cogn.	UPCB	F.A. Michelangeli 1398
76. *Ossaea macrophylla* (Benth.) Cogn.	CAS	Rueda et al. 8682. Nicaragua
77. *Ossaea robusta* (Triana) Cogn.	CAS	Sytsma & Stevens 2210. Panama
78. *Pachyanthus lundellianus* (L.O. Williams) Judd & Skean	CAS	Espinal 164
79. *Tetrazygia urbaniana* (Cogn.) Croizat ex Moscoso	CAS	Judd et al. 5113
80. *Tococa caquetana* Sprague	CAS	Cerón & Neill 2340
81. *Tococa guianensis* Aubl.	UPCB	F. Almeda 9008
82. *Tococa nitens* (Benth.) Triana	CAS	Kral 72037
83. *Tococa quadrialata* (Naudin) J.F. Macbr.	CAS	Díaz et al. 698
84. *Tococa spadiciflora* Triana	CAS	Callejas et al. 2708

Continues.

Table 1. Continuation.

Species	Herbarium	Collector
Microliceae tribe		
85. *Chaetostoma armatum* (Spreng.) Cogn.	BHCB	F.A.O. Silveira 4
86. *Lavoisiera campos-portoana* Barreto	BHCB	F.A.O. Silveira 5
87. *Lavoisiera caryophyllea* Naudin	BHCB	F.A.O. Silveira 6
88. *Lavoisiera confertiflora* Naudin	BHCB	F.A.O. Silveira 7
89. *Lavoisiera imbricata* DC.	BHCB	F.A.O. Silveira 9
90. *Microlicia euphorbioides* Mart.	HUFU	P.O. Rosa 543
91. *Microlicia fulva* (Spreng.) Cham.	BHCB	F.A.O. Silveira 10
92. *Microlicia graveolens* DC.	BHCB	F.A.O. Silveira 11
93. *Microlicia polystemma* Naudin	HUFU	R.Romero 7750
94. *Microlicia sp1*	BHCB	F.A.O. Silveira 13
95. *Microlicia tetrasticha* Cogn.	BHCB	F.A.O. Silveira 12
96. *Rhynchanthera cordata* DC.	BHCB	F.A.O. Silveira 14
97. *Rhynchanthera grandiflora* (Aubl.) DC.	BHCB	F.A.O. Silveira 15
98. *Stenodon suberosus* Naudin	HUFU	G.Pereira Silva 6458
99. *Trembleya laniflora* (D. Don) Cogn.	BHCB	F.A.O. Silveira 17
100. *Trembleya parviflora* (D. Don.) Cogn.	BHCB	F.A.O. Silveira 16
Uncertain Group		
101. *Lithobium cordatum* Bong.	HUFU	R.A. Pacheco et al. 173

Thus, the conventional protocol for embedding was not satisfactory for the analyses of these seeds.

Using T2, the seed structure was well maintained. We observed the integrity of the embryo (Figure 1B), and the thin and thick cell walls in the seed coat were well-preserved (Figure 1E). For the periods adopted here, the use of the Franklin maceration solution (Franklin 1945) after fixation did not interfere with the integrity of the *T. laniflora* seeds, even the seed coat epidermis (see Figure 1E). This was the best result found but was different from that observed with T3. Using T3, it was not possible to obtain anatomical sections of seeds with well-preserved embryos (Figure 1C), and the characteristics of the seed coats were inadequate and similar to those obtained with T1 (Figure 1F). These observations emphasized that softening prior to fixation produces damage to the seed coat and embryo, indicating that this process does not constitute a good procedural option.

For all studied species (Table 1), small changes in the duration of the immersion of the seeds in Franklin solution in the incubator at 60°C ensured satisfactory blocks. This variation in the duration may be due to the qualitative and quantitative characteristics of the seed coats, particularly the phenolic contents. Phenolic compounds are commonly encountered in seed coats, as determined by their detachment by a histochemical test performed with toluidine blue in *T. laniflora* (see Figure 1D-F) and in the other 100 studied species. According to Werker (1997), phenolics and their derivatives are the most widespread secretory materials of seed coats and render seed coats hard and impermeable to water. These characteristics, which are observed in Melastomataceae seeds, limit the infiltration of all HEMA solutions and interfere with the preservation of the embryo (Figure 1A, 1C). Although favoring penetration of the embedding solution, T3 changed the cells of the embryo because the maceration solution modified the cell walls in the seed coat, which enabled its action over the embryo (Figure 1C, 1F). Acetic acid, a component of Franklin solution, is a tissue preservative that moves rapidly into tissues but causes swelling or shrinkage of cells (Jensen 1962). This finding indicates that, when the Franklin solution is applied prior to fixation, cell turgor may be changed, which may affect structural maintenance, as was particularly observed in the embryo of the seeds subjected to T3. Furthermore, acetic acid can soften plant tissues and prevent the hardening caused by fixatives (Jensen 1962). This may be one of the factors responsible for the best results obtained with T2. In this treatment, the material was fixed and then softened with acetic acid and hydrogen peroxide. According to Franklin (1945), acetic acid in the presence of oxidizing agents such as hydrogen peroxide has a delignifying action on wood. Thus, it softens the seed coat in our study.

The use of only the Franklin solution was not sufficient to soften the seed coat of *Cytinus* (Vega & Carmo-Oliveira 2007), as observed in this study for seeds of Melastomataceae. The adoption of Franklin solution combined with 10% glycerin mixed with each alcohol and the use of a 30°C water bath favored the softening of all tested seeds. We highlight

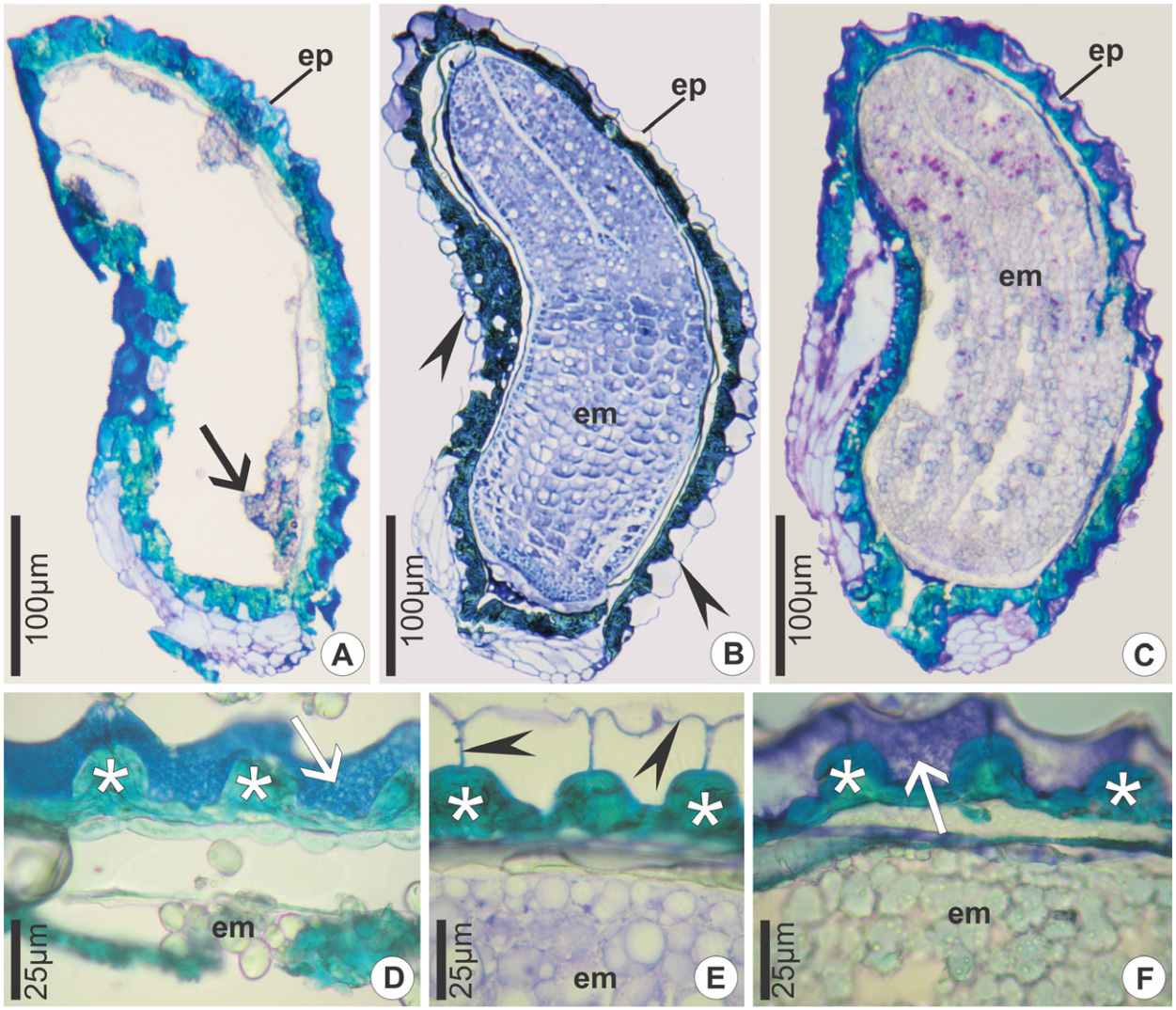

Figure 1. Longitudinal sections of mature seeds of *Trembleya laniflora*. (A-C) General view of the seeds. (A) Treatment 1, control; note the residues of the embryo (black arrow) and the collapsed seed coat cells. (B) Treatment 2, fixation and subsequent softening; note the well-preserved embryo filling the seed and the very good condition of the seed coat (even thin cell walls are preserved, see arrowheads). (C) Treatment 3, softening and subsequent fixation; note the poorly preserved embryo and some changes in the seed coat. (D-F) Details of the seed coats obtained with Treatment 1, 2 and 3, respectively; note the thickened cell walls with phenolic compounds (asterisks), the collapse of the thin walls of the cells in D and F (white arrow) and the visible thin walls obtained with Treatment 2 (arrowheads). (em: embryo, ep: epidermis of the seed coat).

the good results obtained with the Miconieae tribe species (Table 1), which have even thicker seed coats (thick cell walls in several layers).

Franklin solution is traditionally used in studies of wood (Schneider & Carlquist 1997; Chaffey 2002) and for the removal or extraction of fibers (Ericsson & Fries 2004; Kostiainen *et al.* 2006) and other rigid lignified cells that are difficult to analyze by microtomy using conventional plant anatomy techniques. Similar to these types of cells, the seed coat of Melastomataceae presents rigidity, and this finding motivated this study. The option of using Franklin solution in combination with glycerin and slight heating for softening results in a fully satisfactory and inexpensive outcome.

Glycerin is traditionally used as a mounting medium for algae because it has the ability to preserve color and avoid plasmolysis (Johansen 1940). In addition to the preservation effect, it is common in plant microtechnique to use glycerin mixed with alcohol to soften samples, and this prompted the inclusion of this step in our method.

Finally, an increase in temperature is a procedure adopted to break the physical dormancy of seeds (Baskin & Baskin 2000) because the seed coat becomes looser and allows water uptake and gas exchange to the embryo (Yap & Wong 1983). In this study, heating favored softening of the seed coat, thereby enabling appropriate infiltration and embedding into all sections, including the embryo, and thus allowing complete visualization.

Another advantage of using this technique is the low toxicity of the reagents. Jeffrey's method (Johansen 1940) has been previously used to soften *Cytinus* seeds (Vega &

Carmo-Oliveira 2007), but nitric and chromic acid are used in Jeffrey's method (Johansen 1940), both of which are more toxic than acetic acid and hydrogen peroxide.

The methodology developed in this study was proven to be very useful for obtaining sections of small seeds with hard seed coats, such as the seeds in Melastomataceae. We also argue that this protocol can be successfully adapted for use with other organs that exhibit hard features in several plant families, thereby opening great possibilities for its application in a wide range of fields, such as wood anatomy and fruit anatomy and dispersion.

Conclusions

Small hard seeds, which are exemplified by Melastomataceae, are better embedded when they are softened after subjected to fixation. Without softening, the embedding is incomplete, and the sections are of poor quality, making it difficult to observe the cellular features of the seed coat and particularly the embryo. When the softening is performed prior to fixation, the seed coat exhibits specific alterations, and the embryo is not fully preserved. This work establishes a practical, slightly toxic and inexpensive methodology to ensure good preparations for light microscopy that can be helpful in every microtechnique laboratory worldwide.

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