Preparation of Samples for Leaf Architecture Studies, A Method for Mounting Cleared Leaves

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Protocol Note

Preparation of samples for leaf architecture studies, a method for mounting cleared leaves[1]

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- *Premise of the study:* Several recent waves of interest in leaf architecture have shown an expanding range of approaches and applications across a number of disciplines. Despite this increased interest, examination of existing archives of cleared and mounted leaves shows that current methods for mounting, in particular, yield unsatisfactory results and deterioration of samples over relatively short periods. Although techniques for clearing and staining leaves are numerous, published techniques for mounting leaves are scarce.
- *Methods and Results:* Here we present a complete protocol and recommendations for clearing, staining, and imaging leaves, and, most importantly, a method to permanently mount cleared leaves.
- *Conclusions:* The mounting protocol is faster than other methods, inexpensive, and straightforward; moreover, it yields clear and permanent samples that can easily be imaged, scanned, and stored. Specimens mounted with this method preserve well, with leaves that were mounted more than 35 years ago showing no signs of bubbling or discoloration.

Key words: Caroplastic; leaf anatomy; leaf architecture; taxonomy; venation patterns.

Leaf architecture, primarily the study of leaf venation patterns, has been pioneered by paleobotanists, whose primary study material consists of leaf impressions and compressions. However, a survey of the literature on leaf architecture shows several waves of interest in the subject and an expanding range of approaches and applications. These include the evolution of leaf form and function (e.g., Boyce and Knoll, 2002), genetic and other mechanisms in the ontogeny of leaf venation (e.g., Candela et al., 1999), applications in systematics and taxonomy (e.g., Hickey and Taylor, 1991; Fuller and Hickey, 2005), identification for floras and forest management (e.g., Procópio and Secco, 2008; Obermüller et al., 2011), quantitative analyses of leaf venation and other network systems (e.g., Katifori and Magnasco, 2012), and continuing work on paleofloras and paleoecology (e.g., Calvillo-Canadell and Cevallos-Ferriz, 2002; Wing et al., 2009; Gandolfo et al., 2011).

For all of the studies mentioned above, it is essential to have a way to observe in detail even the smallest veins. To this end, a number of clearing methods have been developed (Johansen, 1940; Foster, 1949, 1950; Morley, 1949; Fuchs, 1963; Lersten, 1967; Shobe and Lersten, 1967; Payne, 1969; Pérez-Harguindeguy and Díaz, 2013), but references for mounting and archiving cleared leaves are scarce (Christophel and Blackburn, 1974). For instance, Ellis et al. (2009) and Pérez-Harguindeguy and Díaz (2013) presented simple approaches to clearing and imaging, but mounting was not included.

The traditional mounting media present serious problems for archiving cleared leaves. Glass has desirable optical and archival qualities, but its rigidity usually leads to the formation of bubbles regardless of the sealant used (Fig. 1A, B). Canada balsam, a purified conifer resin, dries very slowly, leading to bubbles; furthermore, over time it shows serious problems with yellowing and crystallization (Fig. 1A). The resin cracking probably results from the loss of solvent from the medium, which oxidizes upon exposure to air or heat. Permount (Thermo Fisher Scientific, Waltham, Massachusetts, USA), a synthetic resin, causes loss of stain and has problems with expanding bubbles (Fig. 1B) as well as crystallization. Some synthetic epoxy resins, such as EPO-TEK 301 (Epoxy Technology, Billerica, Massachusetts, USA), produce bubbles and show problems with cell collapse/shrinkage and loss of stain. Unfortunately, efforts to restore damaged mounted leaves are time-consuming and expensive (Erika González, Smithsonian Institution, personal communication, 2011). Deteriorated mounted leaves, although useful, can affect interpretations of some features of the leaf architecture. This paper presents a method to permanently mount cleared leaves and provides a complete protocol that also details strategies and procedures for obtaining cleared leaves. Compared to traditional mounting methods, this method has the advantages of being relatively fast and inexpensive and of producing clear, thin samples that can easily be imaged, scanned, and stored (Fig. 1C–E). Moreover, after 37 years,

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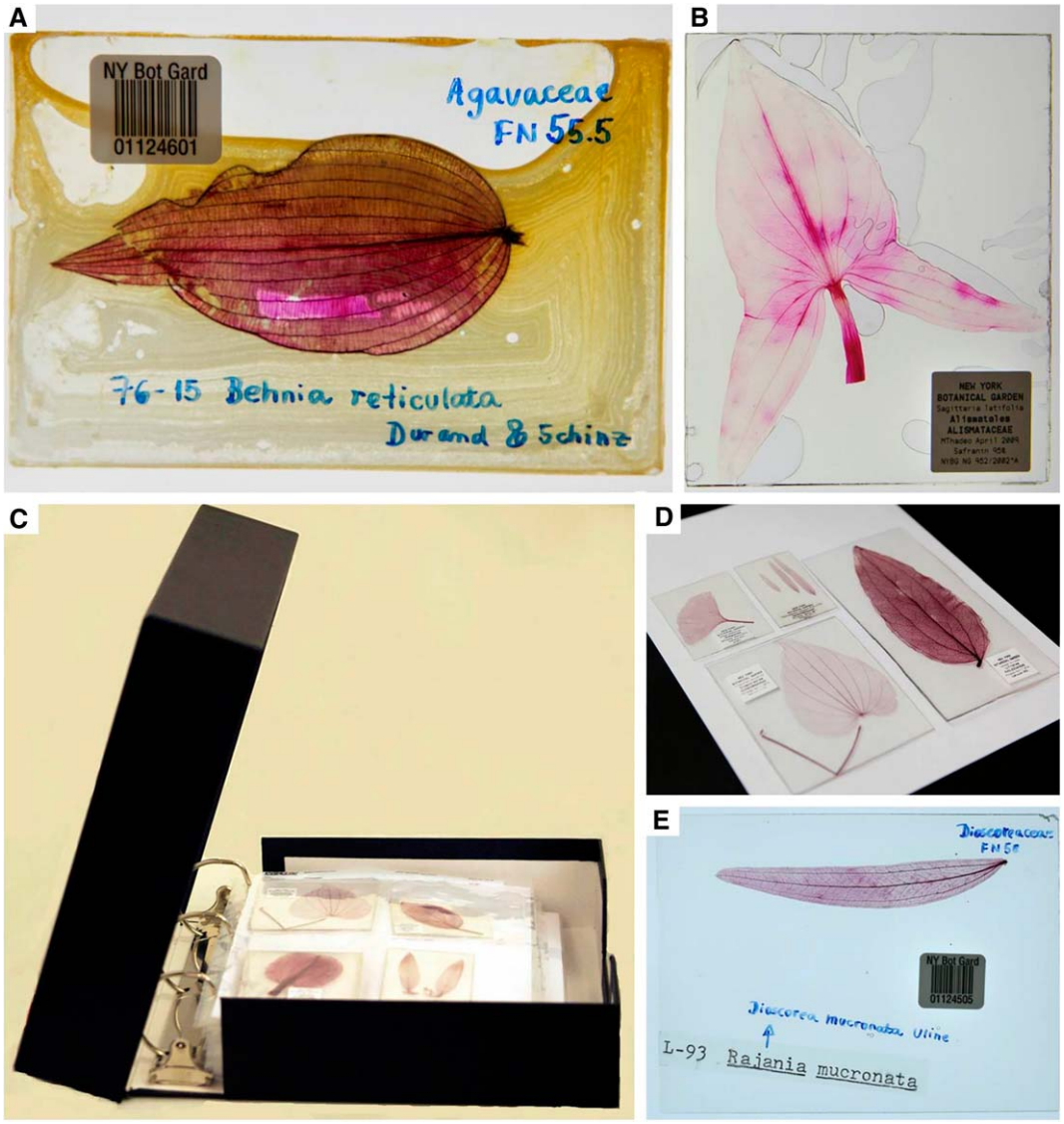

Fig. 1. Cleared leaves from The New York Botanical Garden collection. (A, B) Leaves mounted in glass and traditional mounting media, showing deterioration problems. (C–E) Leaves mounted with the new method in Caroplastic. (A) Leaf mounted in Canada balsam in 1978, showing bubbles, yellowing, and crystallization. (B) Leaf mounted in Permount in 2009, showing bubbles and loss of stain. (C) Archival boxes and plastic sleeves to archive cleared mounted leaves. (D) Leaves mounted during the period 2009–2013. (E) Leaf mounted in 1982 showing no signs of damage.

leaves mounted with this distinctive method do not show any signs of the deterioration typical of the other mounting media mentioned here (Fig. 1E).

METHODS AND RESULTS

The mounting method we present here was developed during three different studies: one on leaf venation patterns of the orders Asparagales and Liliales (Conover, 1982), another on leaf venation patterns of the families Burseraceae and Anacardiaceae (Mitchell and Daly, unpublished data), and another on assembling the evolution of venation patterns in monocots (Thadeo and Stevenson, unpublished data).

The clearing and staining procedures are largely based on the numerous clearing methods available (Johansen, 1940; Foster, 1949, 1950; Morley, 1949; Fuchs, 1963; Lersten, 1967; Shobe and Lersten, 1967; Payne, 1969). A list of basic materials and solutions (with their recipes) that are needed for the clearing and mounting procedures is given in Appendix 1.

***Clearing*—**The method is usually applicable to both dry and fresh leaves. It is impossible to know a priori for a given group of plants whether fresh or dry leaf material will clear better, although normally dry leaves clear better than fresh leaves. For best results, fresh leaves should be kept immersed in 50% ethanol for two weeks before clearing; this removes alcohol-soluble materials like phenolics and chlorophyll, as well as other cell contents. Formalin/acetic acid/alcohol (FAA)–fixed leaves can also be used, but they usually do not clear easily because formalin tends to deposit pigments, which causes the leaf to become very dark and fragile.

Leaves that are cracked, broken, perforated, galled, or spotted with mold are less likely to clear and stain uniformly, and they are more likely to fragment or disintegrate near the damaged areas during clearing. Leaves that were dried at very high temperatures are usually very brittle and are easily damaged in clearing.

Some plant groups present special problems for clearing, staining, and/or mounting their leaves. In our experiences to date, ferns and aquatic monocotyledons tend to be delicate and often disintegrate easily during clearing. To avoid disintegration, special attention should be paid when changing solutions during the clearing and mounting processes. Leaves of Rapateaceae and Bromeliaceae contain large amounts of mucilage, which is difficult to remove. The best way of clearing leaves

of these two families is by putting them directly into a bleach solution. For densely pubescent leaves, we recommend putting them under a dissecting microscope and brushing off hairs with a soft dental brush as much as possible before clearing. If hairs are big enough, tweezers can also be used to remove them. In general, highly coriaceous leaves are problematic, but satisfactory results can often be obtained by increasing the concentration of NaOH (up to 20% in extreme cases) and/or increasing the temperature of the oven during the clearing process up to 55°C.

Summary of steps proposed for clearing leaves:

1. To facilitate changing solutions, put the leaf in a shallow glass container and cover it with nylon screening. Place plastic or stainless steel weights over the nylon screening to keep the leaf flat and to prevent flotation; be careful not to put the weights directly over the leaf (Appendix S1A).

2. Immerse the leaf in a 5% solution of NaOH (Appendix S1B) at 40–54°C (Appendix S1C) for one to several days (the time needed will vary from leaf to leaf). If the solution becomes dark, change it as needed. Always keep the leaf submerged; be careful not to let the leaf become exposed as a result of evaporation of the NaOH solution (Appendix S1D). Once the leaf is transparent but not overly fragile, remove it from the oven and let the solution cool. For the leaf to become transparent (Appendix S1E), it can take from one day to several weeks, depending on the leaf's qualities (e.g., size, thickness, lobing, and chemistry).

3. Rinse the leaf three times with tap water (do not allow the water stream to strike the leaf), then leave the leaf in a water bath at room temperature for at least 10 min.

4. Bleach the leaf in sodium hypochlorite (4.5–5.5%), from 20 s up to 10 min. The length of this step depends on the leaf thickness and chemistry; there is no standard time. Monitor the leaf during this step, paying attention to any sign of damage (mainly along the margin). Once the cleared leaf turns white (Appendix S1F), immediately stop the process by rinsing three times with tap water (again do not allow the water stream to strike the leaf). Keep the leaf in a water bath for at least 10 min.

***Staining*—**We suggest two types of stains, acid fuchsin and safranin. Both will stain the veins and create a differential color that will yield strong contrast and sharp images. Which stain is chosen will depend on what is available or on personal preference, but some leaves will react better to one stain than the other; this can only be ascertained for a given group of plants by trying both stains.

A. Acid fuchsin—

1. Rinse the leaf two times in 50% ethanol.

2. Stain in acid fuchsin for at least 24 h, then decant (stain can be reused but may have to be filtered).

3. Rinse the leaf in 50% ethanol to de-stain the leaf to the desired color level. The amount of time the leaf should be de-stained will depend on the leaf chemistry and the desired degree of contrast.

4. Rinse the leaf in 70% ethanol for 10 min (Appendix S2A).

5. De-stain in 95% ethanol acidified with 3–6 drops of HCl 37%, for 10–30 min or until the mesophyll (not the venation) de-stains to a clear white.

6. Make two changes of 100% ethanol, leaving the leaf submerged for a minimum of 10 min each time. The leaf can be stored for 1–2 wk in 100% ethanol without risk of further de-staining. Before mounting, it is advisable that the leaf be kept in 100% ethanol for at least 24 h, as this makes the remaining tissues firmer. If 100% ethanol is not available, keep the leaf in 95% ethanol, and image and mount as soon as possible before it becomes overly de-stained.

B. Safranin—

1. Dehydrate the leaf in a graded ethanol series (50%, 70%, and 95%) for 30 min.

2. Stain in safranin in 95% ethanol (saturated) for 3 h to overnight.

3. Rinse the leaf in 95% ethanol for 10 min.

4. De-stain in 95% ethanol acidified with 3–6 drops of HCl 37%, for 10–30 min or until the mesophyll (not the venation) de-stains to a clear white.

5. Make two changes of 100% ethanol, leaving the leaf submerged for a minimum of 10 min each time. The leaf can be stored for 1–2 wk in 100% ethanol without risk of de-staining. Before mounting, it is advisable that the leaf be kept at least 24 h in 100% ethanol, as this makes the remaining tissues firmer. If 100% ethanol is not available, keep the leaf in 95% ethanol, and image and mount as soon as possible before it becomes overly de-stained.

***Mounting*—**Caroplastic (Carolina Biological Supply Company, Burlington, North Carolina, USA) is a polyester resin that will harden at room temperature after a catalyst is added. The uncatalyzed resin is soluble only in acetone and thus very difficult to clean off any laboratory equipment. We recommend designating some glass containers for exclusive use with Caroplastic and to always reuse them when mounting leaves. If a fume hood is available, we recommend using it for this part of the procedure; otherwise, make sure the procedure is done in a well-ventilated area.

1. Transfer the leaf from the 100% ethanol to acetone : ethanol 1 : 1 for 30 min.

2. Without allowing the leaf surface to dry, transfer the leaf to 100% acetone for 30 min. Repeat this step (Appendix S2B).

3. Transfer the leaf to freshly made acetone : uncatalyzed polyester resin (Caroplastic) 1 : 1 for 3 h (Appendix S2C).

4. To prepare the final mounting medium, mix the uncatalyzed resin and the catalyst according to the manufacturer's directions: four drops for 30 mL (Appendix S2D). Stir well with a precleaned disposable wooden stir stick for at least 1 min (Appendix S2E) and let it rest for 2–5 min before mounting; that will help avoid bubbles.

5. Remove the leaf from the acetone : uncatalyzed polyester resin bath and drain the leaf of excess resin without allowing the leaf surface to dry (Appendix S2F).

6. Using catalyzed resin as the mounting medium, mount leaves between sheets of write-on acetate. (For details see Appendix S3A–F.) Do not use any other type of acetate, because most of them are porous and the final preparations will be cloudy.

7. Let the mounted leaf dry overnight. Small weights can be put over the acetate to help keep the leaf flat.

8. Place the leaf in a 40–54°C oven for 3 h or longer to promote polymerization. This step speeds up the process, but it can be skipped if a laboratory oven is not available. In that case, let the mounted leaves air-dry for 48 h.

9. Let cool and carefully remove the acetate. Trim excess resin with a fine-point soldering iron or cut plastic with a hot knife or hot scissors (heated with a candle flame). Mounted leaves can be stored in archival boxes within plastic sleeves, keeping them away from light and dust (Fig. 1C).

***Imaging*—**Caroplastic gives excellent, distortion-free viewing and lends itself to the use of magnifying and photographic equipment. However, we still recommend taking digital images before mounting when possible, because there is always the possibility that leaf material can suffer mechanical damage or lose stain during the mounting process. To photograph unmounted leaves, they can be floated in 100% ethanol (to avoid loss of stain) in a large, clear glass container. Cured Caroplastic is also resistant to heat and chemicals, and it has good luminous light transmission of 89.5% and a refractive index of 1.5593 (Edgerton, 2001). When polymerized, Caroplastic is brittle and scratches easily, so it must be handled carefully when dried. Imaging of mounted cleared leaves has traditionally been done with a dissecting microscope paired with a digital camera, using bottom lighting or with lighting plates, but recently other methods have been used (Katifori and Magnasco, 2012).

Using our cleared leaves mounted in Caroplastic, Katifori and Magnasco (2012) made a shallow chamber directly on the glass of a high-resolution flatbed scanner (Epson Perfection V750, Epson America Inc., Long Beach, California, USA), which they filled with index-matching medium (in this case degassed water). They submerged our leaf samples and scanned them at 6400 dpi. This method allowed them to obtain high-resolution images with uniform illumination and minimal sample defects, obviating the need to take multiple images, as the resolution was high enough to zoom in to the finest veins. Moreover, the optical properties of the solution "eliminated" scratches on the Caroplastic. Our Caroplastic-embedded leaf samples did not suffer any damage because of this imaging process.

CONCLUSIONS

The mounting method presented here shortens the drying time of nearly three weeks for mounting media such as Canada balsam and Permount to three days. Based on the evidence available, Caroplastic is in most instances a desirable solution for the long-term storage of cleared leaves, as it yields archival-quality samples that can constitute permanent cleared leaf collections.

Caroplastic preserves well; we have samples from the leaf collection of Conover (1982) that were cleared and mounted in Caroplastic more than 35 years ago, and these show no signs of bubbling or discoloration (Fig. 1E). Caroplastic has the advantage of being lightweight, and the use of write-on acetate sheets (Step 6 of the mounting protocol above) allows very thin preparations;

however, even in thick mounts Caroplastic gives excellent, distortion-free viewing and allows the use of scanners, microscopes, and photographic equipment.

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Appendix 1. List of basic materials and solutions that are needed for the clearing and mounting procedures.

Materials

- Scale
- Glass containers (resistant to moderate heat)
- Nylon screening
- Plastic or stainless steel weights
- Disposable paper cups
- Spatula (metal and wood)
- Write-on acetate
- Plastic dropper

Solutions:

- Ethanol: 50%, 70%, 95%, and 100%
- NaOH: 5–20%
- Sodium hypochlorite: 4.5–5.5% (NaClO, commercial bleach)
- HCl: 37%
- Acid fuchsin 1%: 1 g of acid fuchsin, 1 mL of glacial acetic acid (HOAc), 100 mL of distilled water (Bruce-Gregorios, 1974)
- Safranin in 95% ethanol (saturated): 1 g of safranin, 100 mL of 95% ethanol (Johansen, 1940)
- Caroplastic (sold by Carolina Biological Supply Company, Burlington, North Carolina, USA)

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