Evaluation of distilled water as a mountant in the slide preparation for phytolith identification

Rajalakshmi Karakulam* and K. Krishnan

Department of Archaeology and Ancient History, The Maharaja Sayajirao University of Baroda, Vadodara 390 005, India

Microscopy is a critical component in phytolith research. To identify and count distinct morphotypes, phytoliths extracted from sediments are mounted on microscopy slides and observed under a microscope. The mounting material used to adhere the samples to the slides affects visibility and image quality. Mountants are chosen depending on whether a temporary or permanent slide is required. Benzyl benzoate, microscopy immersion oil, glycerol and distilled water are a few temporary mountants used for phytolith analysis. In the present study, we evaluate the efficiency of distilled water as a temporary mountant with regard to viewing efficiency, image clarity and ability of rotation of phytoliths.

Keywords: Archaeobotany, distilled water, microscopy, mountant, phytolith.

PLANTS produce phytoliths, which are minute casts of cells or gaps between cells that are found in the environment. Compared to the other micro-remains used for studying ancient flora, phytoliths are more resilient as they contain silicon dioxide (SiO_2). When a plant dies, its phytoliths are discharged into the surrounding sediments. Further, they are found to survive the digestion process of organisms and become a part of the faecal matter of animals, and hence also detected in coprolites[1]. Moreover, the preservation of phytoliths is higher in volcanic soils and soils with higher acidic conditions, and lower in saline–alkaline soils[2].

Phytoliths are used in various environmental and archaeological research, and the first step in this regard is their extraction from sediments. Various protocols have been developed for their extraction; the basic procedures involved are sieving, deflocculation, removal of carbonates, removal of organic content and densimetric separation of minerals (heavy liquid floatation)[3]. Once the phytoliths are extracted, they are mounted on microscopic slides and viewed under different magnifications to determine their morphology and distribution. While the identification of morphotypes is possible at a magnification of 250×, higher magnification is required to observe the surface features and measure the different parts. This aids the differentiation of closely related morphotypes.

*For correspondence. (e-mail: rkarakulam90@gmail.com)

The laboratory procedures involved in the extraction process have been discussed in several works, which compare and contrast different approaches, and evaluate them for a greater yield of phytoliths or shorter processing time. Lentfer and Boyd[4] examined the efficiency of centrifugation to remove clay from fine-grained sediments to extract phytoliths, which greatly reduces the extraction time. Zhao and Pearsall[5] altered the sequence of the pre-floatation soil treatments to improve efficiency and reduce extraction time. Lentfer and Boyd[6] compared three different protocols used for phytolith extraction and concluded that the heavy liquid floatation method gives the best results. Horrocks[7] presented a combined procedure to recover phytoliths and starch grains in a single method. Parr *et al.*[8] introduced the microwave digestion technique. Coil *et al.*[9] examined several microfossil extraction methods and provided guidelines for each step dependent on the extraction objective; however, they did not provide a definitive protocol, but only guidelines for tailoring the procedure to different extraction objectives and sediment types.

On the other hand, only a few studies are available on the microscopy aspect of phytolith analyses. Coil *et al.*[9] briefly mentioned different types of mounting media (or mountants) used by researchers without attempting to draw comparisons of their efficacy; also, they did not deal with temporary slide mounting in detail. Hence, it was deemed essential to do a comparative analysis of different types of mountants commonly used for phytolith microscopy to assess their efficacy and arrive at a best-suited method.

Role of mountant: permanent and temporary slides

All methods of microscopy necessitate the use of a mounting medium. It is used to secure samples to slides, prevent them from falling off the slides and also allows for easier observation of the samples under examination. The mounting medium is also required for the storage and transportation of slides for the reference collection. The properties of mountants are critical for image generation since they affect how the specimen is seen through the microscope. The clarity of the image and the capacity to rotate it for easier identification are both critical in the identification and

Table 1. List of commonly used mountants and their properties

Mountant	Refractive index (at 20°C)	Properties	Reference
Permanent			
Canada Balsam	1.54–1.55	Hardens in 24–48 h Optical properties similar to glass Derived from fir tree	13, 14
DPX	1.51–1.52	Dries and hardens with an hour Synthetic non-aqueous Solvent – xylene	17–19
Entellan	1.49–1.50	Synthetic, non-aqueous Dries and hardens in a few hours Solvent – xylene	23
Eukitt	1.49–1.50	Quick drying, dries with an hour Synthetic resin Solvent – xylene	23, 24
Permount	1.51–1.52	Synthetic resin Solvent – toluene Dries within a few hours	7, 21, 22
Temporary			
Benzyl benzoate	1.56	Used as an antiseptic Does not harden	4
Distilled water	1.3	Does not harden	40–42
Glycerol	1.47	Does not harden Used for pollen microscopy	32, 33
Immersion oil	1.51	Used as a connection between objective lens and cover slip Does not harden	39

statistical analysis of phytoliths, and thus, are of paramount importance while discussing the choice of mountants. Several mountants are available to an archaeobotanist, each with its own properties and applications.

In phytolith microscopy, researchers require two forms of slide preparation – permanent and temporary. Permanent slides are prepared to compile a reference collection of various morphotypes of phytoliths for future use. The mountant for a permanent slide should harden to become a solid either with time or when exposed to high temperature. Another method of making a permanent slide is to use a sealant around the coverslip to keep the mountant in place and prevent it from drying. Natural and synthetic resins are the common choice of mountants (Canada Balsam, Entellan and Eukitt) in permanent slide preparation. These remain as fluids for a brief period, allowing phytolith rotation. The time can range from a few hours to several days, depending on the mountant as well as the temperature of the surroundings.

The alternative method of slide preparation that is required for phytolith microscopy is the temporary one. For quick viewing, identifying and counting phytoliths, a temporary slide is preferred. These cannot be used to store sample slides as the mountant does not harden. Benzyl benzoate, glycerine, microscope immersion oil and distilled water are examples of mountants commonly used for temporary slides.

Mountants used in phytolith microscopy

In the aforesaid context, a review of the published literature indicates that a range of mountants have been used for slide preparation. A review of mounting media used in histopathology and immunochemical staining has been done by Ravikumar *et al.*[10]. Table 1 provides a list of commonly used mountants in phytolith microscopy, along with their details. This may not be considered a comprehensive list. Additionally, several articles entirely omit to mention the name of the mountant.

Permanent slides

Canada balsam (also known as Canada turpentine or balsam of fir) is an oleoresin produced by the balsam fir of North America, *Abies balsamea*, and is a viscous yellowish to greenish liquid. It hardens as a clear mass and is used as a cement in a variety of applications, including microscopy for mounting specimens and optical work for glass mounting[11]. The optical properties of Canada balsam are nearly identical to those of glass. Its refractive index (RI) is 1.55. It has been used as a mounting medium for phytolith microscopy in several studies[12–14]. Although it is a mountant that hardens into a solid, the time taken (24–48 h) is sufficient to allow observation of phytoliths by rotation. A quick mounting medium, Entellan, with RI of 1.49–1.50, has been used to prepare permanent slides[15]. It is best suited for completely dry samples. Once cured and set, it can survive long periods without cracking or darkening. Dibutylphthalate polystyrene xylene (DPX), a combination of distyrene and xylene, is commonly used for histological studies. It is a synthetic, fast-drying mountant with RI between 1.51 and 1.52 at 20°C (ref. 16). DPX has been used

in phytolith analysis by several researchers[17–19]. Another synthetic resin mountant, Permount, has also been used for phytolith analysis[20–22]. It is a toluene-based resin which has an RI of 1.52. Eukitt with an RI of 1.49 has proven to be a fast-drying mountant. It can withstand the presence of water and trace amounts of alcohol in the sample without compromising on the optical clarity of the images. There are a few works that cite the use of Eukitt[23–25].

Temporary slides

Benzyl benzoate ($C_{14}H_{12}O_2$) is an organic compound used as an antibiotic ointment to treat skin infections[26]. The RI of benzyl benzoate is 1.56 (ref. 27) and it is a clearing agent used in tissue microscopy. In phytolith microscopy, benzyl benzoate has been used for mounting[4,28,29]. Another commonly used mountant is glycerol ($C_3H_8O_3$) or glycerine with an RI of 1.47 (ref. 30). It is commonly used in pollen microscopy because the RI of pollen ranges from 1.55 to 1.60, and glycerine provides good contrast[31]. It has been used in phytolith research, allowing phytolith rotation by pressing gently on the coverslip with a probe[32–34]. However, the downside of utilizing glycerine as a mounting medium is that phytoliths placed in it can be difficult to detect using only brightfield microscopy[35]. RI of 1.42–1.43 has been observed in phytoliths[36]; therefore, there is not much contrast between the two substances. Immersion oil (or microscopy immersion oil) is typically utilized to make a connection between the objective lens and coverslip. It improves the resolving power of the microscope by filling up the space created by the air gap between the two lenses. Its RI is 1.51 (ref. 9), which is higher than that of air and similar to glass (coverslips). Immersion oil has been used as a mountant for phytolith analysis[37–39]. It does not solidify and hence the edges of the coverslip must be sealed in order to make permanent slides. The sealing can be done by applying transparent nail polish on the edges of each coverslip and letting it dry completely.

A review of the literature revealed that the use of distilled water had only been mentioned in a few studies[40–42]. It was employed for rapid mounting and viewing. Since it is also easily available and inexpensive, the use of distilled water in phytolith microscopy should be examined.

Materials and methods

The procedure used for preparing phytolith slides for microscopic observation is described here. The extracted phytolith samples were dried and stored in vials. First, a drop of the mountant was placed on a clean, labelled microscopic slide (Blue Ribbon microscope slides, 1.35 mm thickness). The sample was then dispersed in the mountant with a probe, followed by placing the coverslip (Blue Ribbon microscopic cover glass, Grade 1, English glass) on it. The prepared slides were observed using a bright field microscope (Leitz-Laborlux 12 Pol-D) at a magnification of 250×. To qualitatively differentiate the efficacy of different mountants, experimental samples were prepared using Canada balsam, benzyl benzoate, glycerol, microscopy immersion oil and distilled water (laboratory-grade). Canada balsam was included in the experiment, though it is a permanent mountant, as it does not solidify for 24–48 h, thus allowing the movement of phytoliths for observation. Both dry samples as well as wet samples were used in the experiment.

It was observed that in the case of dry samples dispersed in Canada balsam, the images were not clear and the phytoliths had an ill-defined outline (Figure 1 *a*). Benzyl benzoate gave comparatively better results with the dry sample, albeit the images were unclear with indistinct outlines (Figure 1 *c*). In comparison to benzyl benzoate, glycerol produced less clarity. The appearance of phytoliths was obscure and hence not discernible (Figure 1 *e*). With immersion oil, it was observed that the images were, to a certain degree, clearer than other mountants (Figure 1 *g*). For all the above-mentioned mountants, with the exception of glycerol, it was observed that the particles tended to clump together when wet samples were used in place of dry samples (Figure 1 *b*, *d* and *h*). In the case of glycerol, not much difference was noticed in the quality of images (Figure 1 *f*).

In the experiment, distilled water was the only mountant that gave clear images (Figure 2). The outline of individual phytoliths was clearly visible and the images had a good contrast which aided in the identification and counting procedure. It was observed that for the preparation of temporary slides for training and practice in the identification of phytoliths, distilled water was the best mountant. It is easily available and cost-efficient. Another advantage is that the phytoliths do not need to be completely dried before being mounted on slides, which reduces the processing time for phytolith analyses. In the case of any other mounting medium, it has to be ensured that the phytoliths are completely dry. The experiment was conducted at room temperature (30°C) at a 50% humidity level (air-conditioned laboratory without the use of a fan), which gave approximately 2 h for observation of a single slide. Beyond 2 h, the mountant (distilled water) began shrinking from the edges. This time can be extended to approximately 3.5 h by sealing the edges of the coverslip using transparent nail polish. Moreover, it is observed that if unsealed, the entire slide dries up in 4–4.5 h. This time may vary depending on the room temperature as well as the humidity level of the surroundings.

Discussion

We assessed the significance of clarity, definable outline, ability to rotate the phytoliths and the role of RI of the mountant.

Clarity

Phytoliths are mostly transparent in nature. Some studies have attempted to stain them for better viewing. Dayanandan

et al.[36] stained phytoliths in the leaves for better viewing, but in vain. The staining technique has also been used for the archaeological assemblage of phytoliths[43], but not much progress was made. Hence, phytolith researchers deal with transparent objects that cannot be stained. The ability to identify and count them depends on image clarity. Blurry images lead to errors in the counting and identification of phytoliths.

Well-defined outline

In order to identify phytoliths, it is necessary to distinguish their shapes, which is of critical importance. However, in general, if the outline is not clear, it may be difficult to distinguish between two similar morphotypes as well as various varieties of crosses (Figure 3).

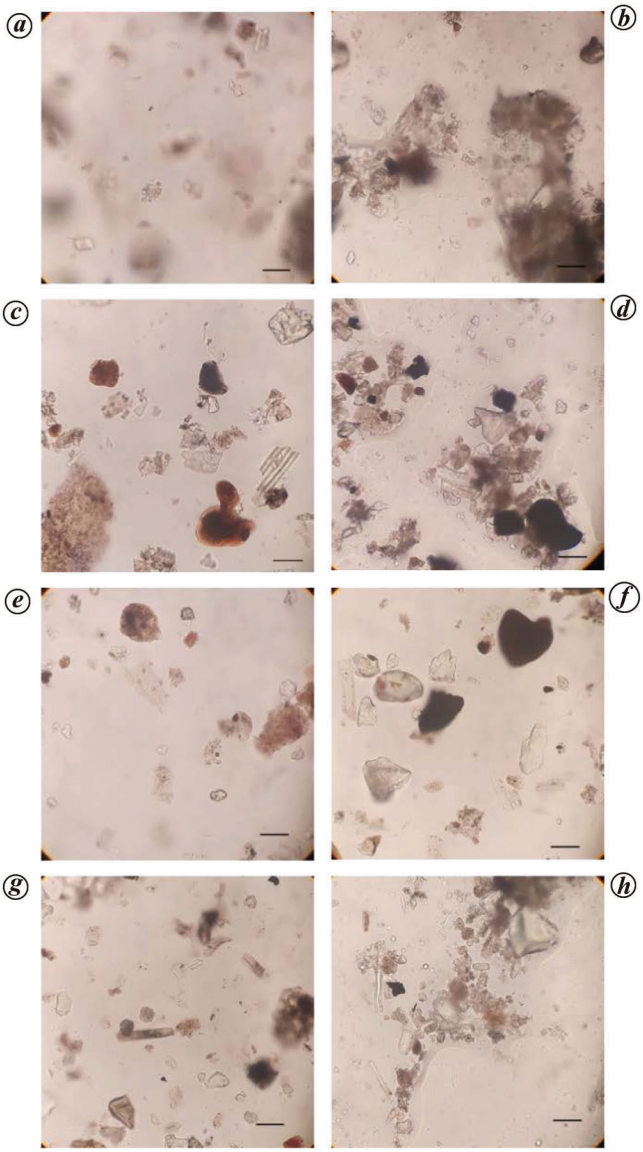

Figure 1. Phytoliths (***a***) dry and (***b***) wet mounted in Canada balsam. Phytoliths (***c***) dry and (***d***) wet mounted in benzyl benzoate. Phytoliths (***e***) dry and (***f***) wet mounted in glycerol. Phytoliths (***g***) dry and (***h***) wet mounted in immersion oil. (Scale bar = 40 µm.)

Ability to rotate the phytolith

As phytoliths are three-dimensional particles, it is best to observe them from all directions for proper identification. By gently tapping the coverslip, one can rotate the phytoliths and observe the features from all angles. This is especially crucial to differentiate morphotypes which might have similar features on a two-dimensional plane, but are different when observed from a side view. For example, Pearsall *et al.*[44] suggested that rotating the phytoliths helped avoid confusion between wavy-top and three-spiked rondels.

Role of refractive index of the mounting medium in microscopy

Any object embedded in a medium has to bend the path of light as it travels through a boundary between two media. The clarity or contrast of phytoliths results from different RIs of separate media. If RIs of the two media are the same, light passes through them without bending, essentially making the object invisible[45] (Figure 4, case II). As the difference in RI increases, refraction increases and light moves away from the imaging lens, darkening the edges (Figure 4, case I).

In general, this method is found extremely useful in preparing temporary slides, where transparency plays a key role. RI of phytoliths falls in the range of 1.47–1.48. Largely, the level of hydration present in silica determines

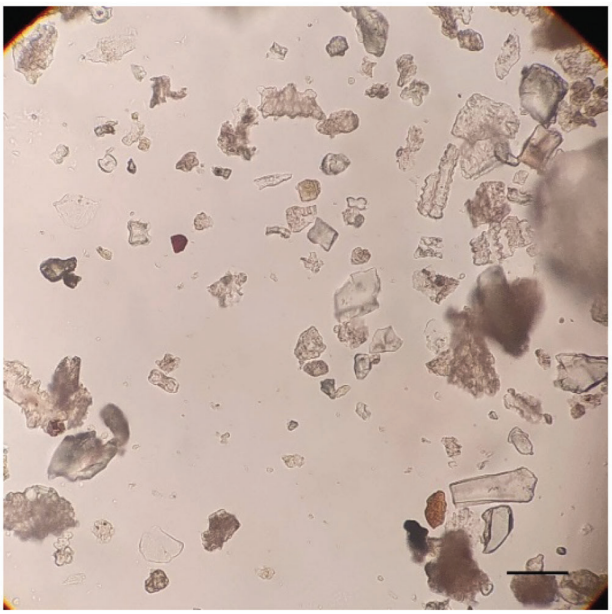

Figure 2. Phytoliths mounted in distilled water (scale bar = 40 µm).

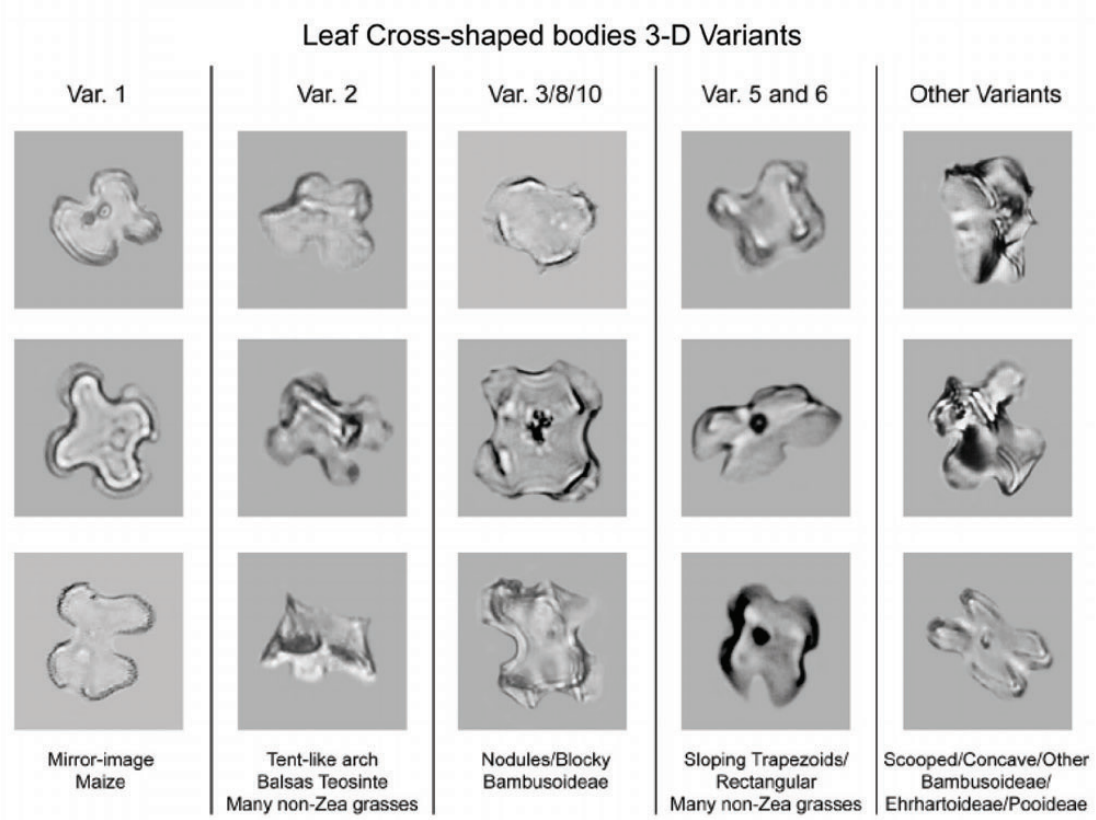

Figure 3. Varieties of cross-shaped phytoliths found in grasses (image courtesy: Piperno[3]).

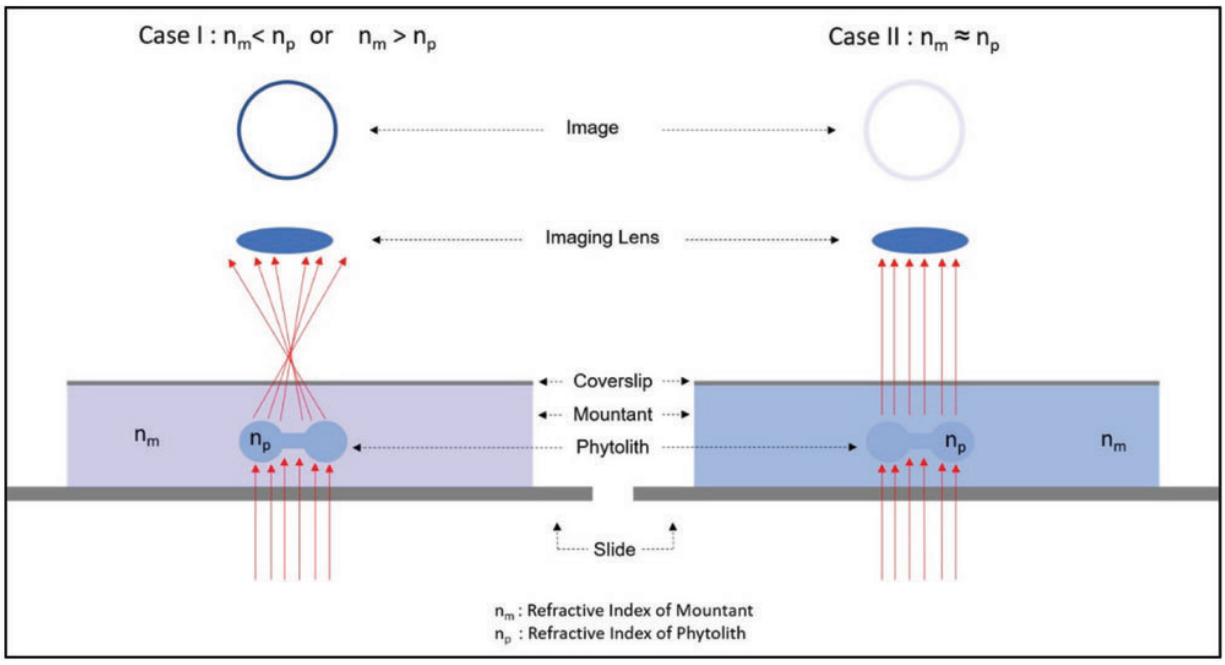

Figure 4. Diagram illustrating the passage of light through different media.

its RI[46]. In fact, the difference in RI of hydrated and dehydrated silica in phytoliths has been used to differentiate between assemblages that have undergone the burning process and those which have not[47]. RI of 1.47 ensures that when mounted in distilled water (RI = 1.3), the images have good contrast and are clearly visible.

Conclusion

Andersen's[48] properties of an ideal mounting medium are primarily for pollen grains. Adopting these for phytolith microscopy, the following criteria must be considered:

(i) RI of the mountant should differ substantially (but not greatly) from that of the phytolith.
(ii) Mountant should not dry up fast and stay in fluid form long enough to identify and count phytoliths.
(iii) Mountant should not be volatile.
(iv) Mountant should be inert and not react with silica.
(v) Mountant should be commercially inexpensive and easily available.

The findings of the present study reveal that using distilled water as a mountant is ideal when temporary slides are prepared for the identification of phytoliths. Due to the appropriate difference between RI of distilled water and phytoliths, the former is best suited for imaging purposes. Further, it is readily available and inexpensive. Distilled water is particularly effective for training students and early researchers in laboratory methods. Unlike other mounting procedures, it does not require the samples to be totally dry prior to mounting and allows for rotation of the phytoliths for viewing from all directions.

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