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Application of acetolysis in phytoliths extraction

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ABSTRACT

Phytoliths are inorganic particles derived from plants, which can be applied in several areas such as plant taxonomy, systematic and paleontological studies. Dry and wet ashing are employed in phytolith extraction from plant tissues and soil. Although they are both well established and widely applied in the phytolith morphological analysis, they can be inefficient to fully remove the organic matter. To overcome this problem, we evaluate the palynological method, acetolysis, for extracting phytoliths. Leaf fragments of *Mourera fluviatilis* Aublet, a species of the rheophytic family Podostemaceae, was tested with two variables: temperature and time. The obtained protocol was employed in other twelve species. The samples were analyzed in light and scanning electron microscopy. Our results indicate the efficacy of acetolysis in isolating phytoliths from botanical samples, providing a clear surface to the detailed analysis on its ornamentation.

1. Introduction

Phytoliths or silica bodies are hydrated silicon dioxide particles ($SiO_2.H_2O$), commonly known as biogenic silica, which precipitate and are deposited in tissues of different organs in many groups of non-vascular and vascular plants (Williams, 1986; Epstein, 1999). Their morphology has provided relevant insights to several areas, such as plant taxonomy, paleobotany and paleoclimatology (e.g., Prychid et al., 2004; Dickau et al., 2013). In order to access the phytolith morphology, the surrounding organic material must be dissolved or removed from the sample. Assorted techniques have been employed to expose or to extract phytoliths from plant tissues. These techniques are commonly grouped into two main categories: dry ashing and wet ashing (Rovner, 1983).

Dry ashing was the first method applied to detach phytoliths from the surrounding organic matter, by the French chemist Raspail (1839) in the analysis of *Spongilla*, a genus of freshwater sponges. Subsequently, several researchers have successfully employed this method to investigate the ash content of fossil (e.g., Netolitzky, 1914) and extant (e.g., Molisch, 1920) plants. The dry ashing technique basically applies high temperatures as a means to oxidize organic material. The samples are placed in porcelain crucibles and ignited in a muffle furnace. Incineration temperatures are generally maintained between 450 °C and 900 °C (Jones and Handreck, 1967). A post-treatment with hydrochloric acid (HCl) and nitric acid (HNO_3) may be required to remove residual material (Jones and Milne, 1963; Theunissen, 1994).

The wet ashing technique was proposed by Miliarakis (1884), who used sulfuric acid and a 20% aqueous solution of chromic acid. Later, this technique was widely recognized (Parry and Smithson, 1957; Jones and Milne, 1963; Rovner, 1972). At the present time, different types of main oxidizing solutions are used, such as nitric acid and perchloric acid (Rovner, 1971; Hayward and Parry, 1980) and sulfuric acid (Geiss, 1978). Samples may be pre-treated with hydrochloric acid and/or post-treated with it or hydrogen peroxide (see Corbineau et al., 2013 for more details).

Albeit these two techniques and their variations (e.g., microwave digestion; Parr et al., 2001) are broadly applied for phytolith extraction, some researchers have, however, reported a few problems. Jenkins (2009) found greater percentages of conjoined phytoliths in fresh samples of *Triticum durum* Desfontaines (wheat) treated by dry ashing than in those treated by wet ashing. Moreover, the wet ashing process can consume different amounts of time depending on the sample (Lu and Liu, 2003) or cannot even be fully efficient to digest the organic matter (Jenkins, 2009; Kameník et al., 2013).

Since the acetolysis method was introduced by Erdtman (1952, 1960), it has been extensively used in pollen analysis with light and electron microscopes. In essence, acetolysis involves treating pollen samples in a 9:1 solution of acetic anhydride ($C_4H_6O_3$) and sulfuric acid (H_2SO_4) heated, which dissolves all non-sporopollenin substances (cell contents and pollenkitt), permitting the detailed investigation of the outer wall of plant spores and pollen grains.

Podostemaceae, or riverweeds, is an aquatic angiosperm family growing submerged on solid substrate (generally rocks) in fast-

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flowing water. Recently the phytoliths were considered to be informative taxonomic characters (Costa et al., 2011) and we are conducting research to evaluate whether they can also provide information on the phylogeny. Nonetheless, we have experienced some difficulties in applying dry or wet ashing techniques to extract phytoliths from vegetative shoots; i.e., we tried a dry ashing technique in muffle furnace at 600 °C for six hours and the obtained result showed plenty of organic matter covering the entirely phytolith surface, making their analysis difficult (Plate I, 1). To overcome this problem, we aim to evaluate the efficacy of the acetolysis technique for the phytolith extraction from stems and leaves (Plate I, 2).

2. Material and methods

We randomly designated *Mourera fluviatilis* Aublet (Podostemaceae) as model species to establish optimal conditions of temperature and time to the proposed methodology. In order to verify the consistency of the obtained protocol, we replicated it in twelve other species of the same family, using one specimen from each species (Table 1).

Leaf fragments used in this study were fixed in FAA_{70} and stored in 70% ethyl ethanol. The acetolysis method, adapted from Bennet and Willis (2002), was employed as the following (centrifugation conducted at 2000 rpm for 10 min):

1- Place the leaf samples (approximately 1 cm^2) in Falcon tubes with 2 cm^3 of glacial acetic acid and soak to homogenize. After 24 h, centrifuge and decant.
2- Add 2 cm^3 of acetolytic mixture (acetic anhydride and sulfuric acid; 1:1) and place it in a water bath.
3- Add 2 cm^3 of distilled water with a few drops of acetone, centrifuge and decant.
4- Add 2 cm^3 of glycerin water (for light microscopy analysis) or 70% ethyl ethanol (for scanning electron microscopy).
5- Mounting:
   (a) For light microscopy, mount with glycerin jelly (according to Kisser, 1935).
   (b) For scanning electron microscopy, place the material on aluminum stubs and then sputter coat with gold–palladium.

To identify the required conditions to produce clean-surface phytoliths, we investigated two variables: temperature and time. Firstly, samples were acetolysed in water bath in different conditions of temperature: at room temperature (c. 24 °C) for 2 min, room temperature increasing up to 70 °C for 2 min, 90 °C for 1 min and 100 °C for 1 min. Then, after determining the temperature that provided the cleanest surface, we increased the time by 5 and 10 min in order to verify the influence in the dissolution of the residual organic matter. The better conditions of temperature and time were applied to replicate acetolysis in specimens of the twelve other species.

Table 1
List of neotropical Podostemaceae species analyzed, respective vouchers and herbaria.

Species	Voucher (herbaria)
Apinagia longifolia (Tulasne) P. Royen	C. P. Bove et al. 1943 (R)
Apinagia staheliana (Tulasne) P. Royen	C. P. Bove et al. 1869 (R)
Ceratolacis pedunculatum C.T. Philbrick, Novelo et Irgang	C. P. Bove et al. 2334 (R)
Cipoia inserta C.T. Philbrick, Novelo et Irgang[b]	C. P. Bove et al. 2317 (R)
Cipoia ramosa C.P. Bove, C.T. Philbrick et Novelo [b]	C. P. Bove et al. 2251 (R)
Diamantina lombardii Novelo, C.T. Philbrick et Irgang	C. P. Bove et al. 2131 (R)
Mourera aspera Tulasne	C. P. Bove et al. 2260 (R)
Mourera fluviatilis Aublet[a,b]	C. P. Bove et al. 1944 (R)
Noveloa coulteriana (Tulasne) C.T. Philbrick	C. P. Bove et al. 1631 (R)
Podostemum ceratophyllum Michaux	C. T. Philbrick et al. 4634 (R, WCSU)
Podostemum scaturiginum (Martius) C.T. Philbrick et Novelo	C. P. Bove et al. 2245 (R)
Tristicha trifaria (Willdenow) Sprengel	C. P. Bove et al. 1867 (R)
Weddellina squamulosa Tulasne	C. P. Bove et al. 1862 (R)

[a]Model species; [b] Species illustrated in this study.

In light microscopy (LM), samples were analyzed at 400× and 1000× magnification. Images were captured with an Olympus BX-51 microscope with the image-capture Q color5 and Image-Pro Express software. For scanning electron microscope (SEM), samples were examined on a Jeol JSM-5800, operating at 15 kV.

3. Results

The acetolysed leaf fragments of the model species, *M. fluviatilis*, showed the following results regarding to the temperature. At room temperature, phytoliths can be detected but they remain almost entirely covered by organic matter (Plate II, 1). At room temperature up to 70 °C, they become partially evident but their shape is still obscured (Plate II, 2). At 90 °C some phytoliths become partly exposed and organic matter becomes more fragmented (Plate II, 3). At 100 °C, they are better exposed but few portions of organic matter are still present (Plate II, 4). It is possible to observe a decrease of organic matter related to increasing temperature. Because of the better results obtained at 100 °C, we chose this temperature to evaluate the influence of time on the digestion of the organic matter. Heating at 5 and 10 min gave equally satisfactory results with the phytolith surfaces being completely clean (Plate II, 5, 6).

We applied the acetolysis method at 100 °C for 5 min to the remaining species listed in Table 1 and analyzed the results with LM and SEM (Plate III). Similar effects were obtained, except for *Cipoia inserta* C.T. Philbrick, Novelo et Irgang, *Cipoia ramosa* C.P. Bove, C.T. Philbrick et Novelo and *Diamantina lombardii* Novelo, C.T. Philbrick et Irgang

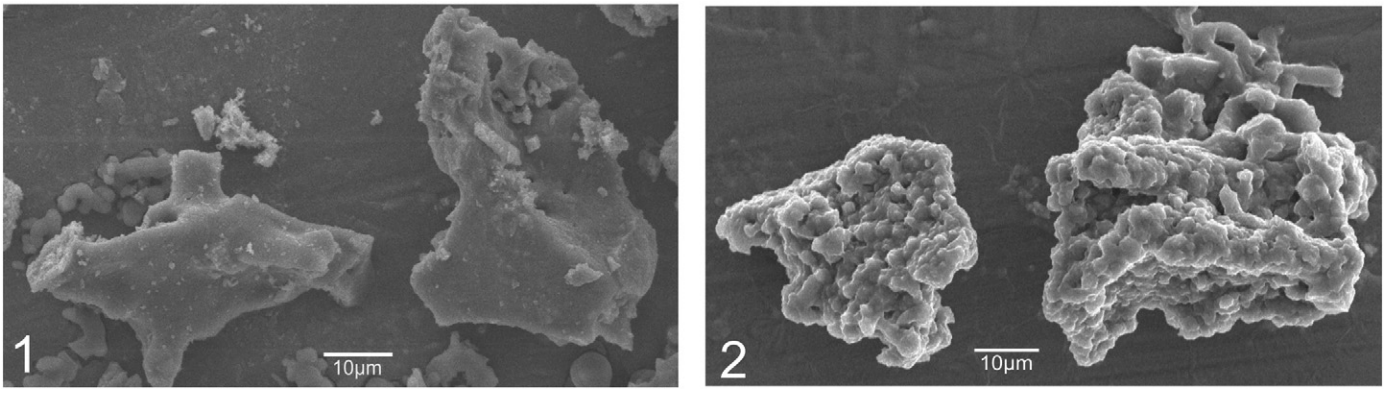

Plate I. Scanning electron microscopy of phytoliths from *M. fluviatilis* (1) under dry-ashing treatment (note organic matter covering the surface) and (2) adapted acetolysis treatment (note verrucose ornamentation revealed).