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Phytolith extraction and counting procedure for modern plant material rich in silica skeletons V.2

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Abstract

Modern plant tissues are often processed for phytolith analysis. They represent a fundamental source of comparison for archeological and palaeoenvironmental phytolith assemblages; they efficiently serve for morphological studies of phytolith shapes and dimensions and, in the last two decades, they have been increasingly involved in physiological studies, which aim to understand the functioning of Si absorption in plants. Here we present a relatively fast, safe, and inexpensive phytolith extraction, combining a dry ashing technique followed by wet oxidation, and a counting methodology. This protocol offers an optimized strategy that achieves very pure samples, preservation of a high number of silica skeletons (phytoliths in anatomical connection), and a counting method which assures the richness and the evenness of the phytolith assemblage distribution. The methodology described in this paper is optimal for recognition and identification of morphotypes, isotope studies and concentration evaluations. This protocol has been developed to allow researchers to extract phytoliths from modern tissues of leaves, stem and chaff, and it is combined with a new strategy to count phytoliths in slides where several big silica skeletons (>50/100 cells each) are present.

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Materials

Consumables:

-Glass beakers (100 ml).
-Crucibles (20 ml) that can stand at least 500ºC in the furnace and equipped with lids.
-Eppendorf tubes (5 ml).
-Sterile centrifuge tubes (5 ml) and racks to store them.
-Microscope slides and covers.
-Micro spatula or micro spoon.

Chemical products:

-Hydrochloric acid 10% v/v.
-Hydrogen peroxide10% v/v.
-Entellan New® (mounting medium).
-Ethanol10% v/v for cleaning tools.
-Distilled water.

Equipment:

-Precision scale (at least 0.0001 g).
-Sonicator (ultrasonic bath).
-Muffle furnace that can reach at least 500ºC.
-Pipette (1-100µl).
-Fume hood.
-High speed centrifuge (6000 rpm) for tubes of 5 ml.
-Laboratory drying cabinet (30-60ºC).
-Optical microscope. The optimum setting includes a digital camera for photographing phytoliths. A good quality scanning can be done with a 400-600x magnification.

Safety warnings

Label elements, including precautionary statements

Hydrochloric acid
(CAS-No.)7647-01-0
(EC-No.)231-595-7;231-596-7
(EC Index-No.)017-002-01-X
(REACH-no)01-2119484862-27

H314 - Causes severe skin burns and eye damage.
H335 - May cause respiratory irritation.

P301+P330+P331 - IF SWALLOWED: rinse mouth. Do NOT induce vomiting.
P303+P361+P353 - IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water/shower.
P304+P340 - IF INHALED: Remove person to fresh air and keep comfortable for breathing.
P305+P351+P338 - IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P310 - Immediately call a POISON CENTER/doctor.

Hydrogen peroxide
(CAS-No.)7722-84-1
(EC-No.)231-765-0
(EC Index-No.)008-003-00-9
(REACH-no)01-2119485845-22

H271 - May cause fire or explosion; strong oxidiser.
H332 - Harmful if inhaled.
H302 - Harmful if swallowed.
H314 - Causes severe skin burns and eye damage.

P210 - Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking.
P280 - Wear protective gloves/protective clothing/eye protection/face protection.
P301+P330+P331 - IF SWALLOWED: rinse mouth. Do NOT induce vomiting.
P304+P340 - IF INHALED: Remove person to fresh air and keep comfortable for breathing.
P310 - Immediately call a POISON CENTER/doctor.

Entellan® New
(CAS-No.) 1330-20-7
(EC-No.) 1272/2008

H226 Flammable liquid and vapour
H312 + H332 Harmful in contact with skin or if inhaled
H315 Causes skin irritation

P210 Keep away from heat.Response
P302 + P352 IF ON SKIN: Wash with plenty of soap and water
P304 + P340 IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing

Before start

We tested the protocol on monocots tissues, which produce high concentrations of phytoliths in a reduced volume. However, the dimension of the glassware, crucible and tubes can be adapted to the plant material of interest.

The protocol takes up to 10 days to complete, from when plant tissues are collected from the plant to when samples can be observed at the microscope and depending on the plant material and humidity conditions. Skipping the first drying steps (tips 1-2) allows for faster extractions. The number of samples that can be processed at one time depends on the laboratory space (mostly the furnace cabinet space and the centrifuge) and the experience of the practitioner.

Drying plant material

1

Note

The first steps (points 1 and 2) aim to obtain very clean and dry samples to evaluate biomass production before the extraction and to store plant tissues for future use. If biomass evaluation or storage are not needed, start directly from point 3.

Collect the tissues of interest from the whole plant. Store each sample in a separate paper bag and put the paper bags in a dryer. Paper bags prevent the formation of fungi and bacterial infection, allowing the evaporation of tissues' humidity.Collect the tissues of interest from the whole plant. Store each sample in a separate paper bag and put the paper bags in a dryer. Paper bags prevent the formation of fungi and bacterial infection, allowing the evaporation of tissues' humidity.

2 Leave the plant tissues to dry at 60-70°C in a dryer (we use a IWC125 Leec drying cabinet). Check the bags once a day to be sure they do not develop any fungi infection because of the heat. Weigh the samples until no weight loss is observed to be sure to have obtained completely dry tissues. Our dried samples weigh on average 45% less of the fresh biomass. Considering that each species/treatment and tissue has its own level of humidity, we suggest testing the tissues for their consistency to make sure they are dry: they will be completely dry when they become brittle (try to crush the leaves with your hands to check their consistency).

3 Wash samples in an ultrasound bath (we used a Ulsonix Proclean 3.0) at room temperature for 00:05:00 to remove extraneous debris (such as soil particles). To wash many samples simultaneously, use small glass beakers: put each sample in a labeled beaker and cover it with distilled water. Put all the beakers in the ultrasound bath and fill the container with water while paying attention not to overflow in the beakers. Cut the samples into pieces to fit into the beakers. 100 ml beakers are large enough to contain samples of grasses. Calibrate beaker and sample sizes based on the species under analysis. 5m

Multiple samples in the ultrasound bath using glass beakers.

4 After 5 minutes, remove the samples from the beaker with a tweezer and place them on aluminum foil to dry.

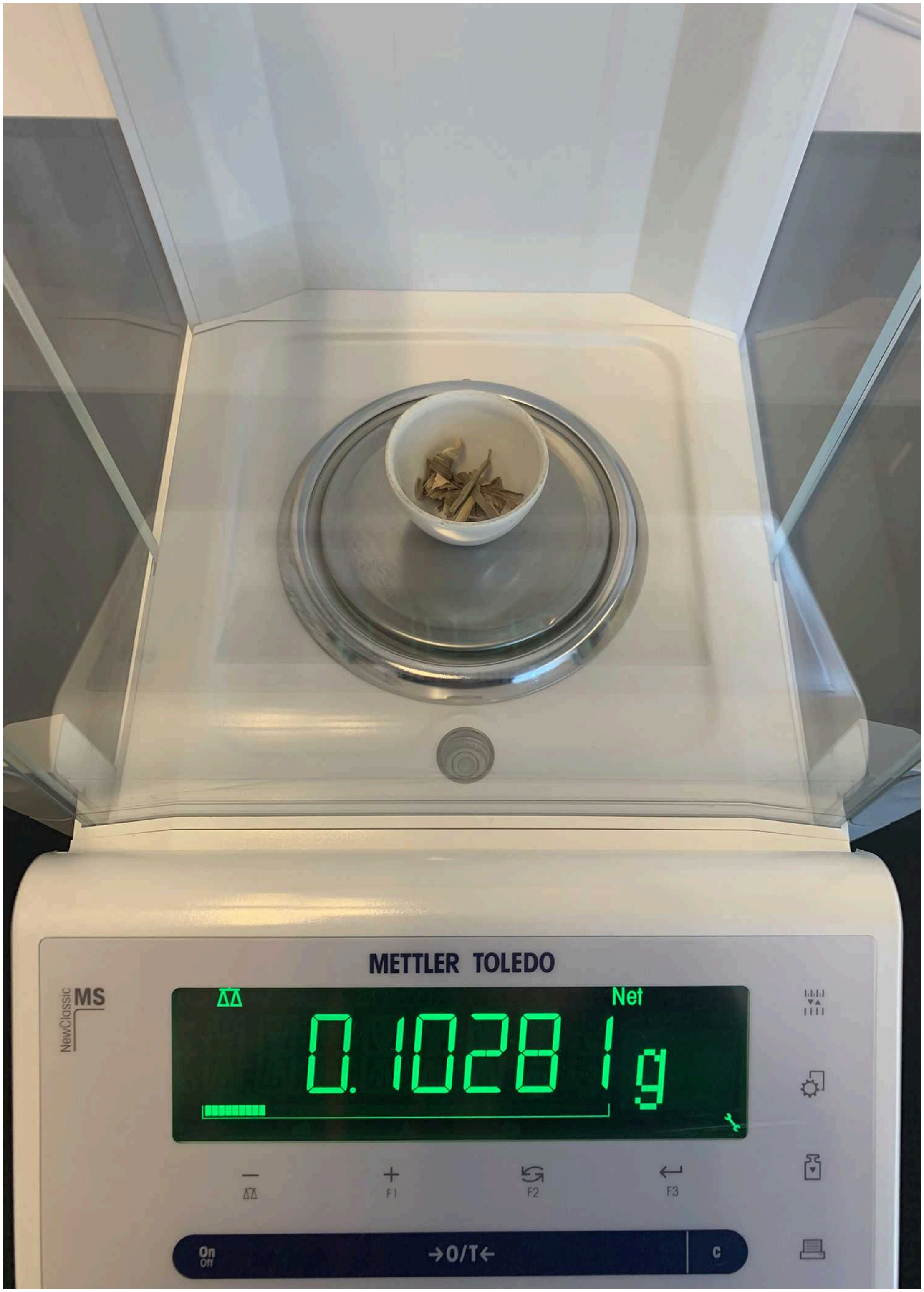
Ashing procedure

5 0.1 g

Note

The following steps ensure the complete decomposition and mineralization of the organic material.

Weigh the plant material (in our case we use a Mettler Toledo MS105DU to weight ca. 0.1 g) in a labeled ceramic crucible



Plant material weighed with a precision scale inside the ceramic crucible.

6 Remove the crucible and cover it with a lid (ceramic lid or foil lid). Do not worry if the samples are not completely sealed by the lid, if oxygen passes inside it will avoid

12h

carbonization. Place the crucibles in the furnace before turning it on and ash them at 500ºC for 12:00:00 (we use a Nabertherm C450 of 5 l capacity). The 12 hours include the time the oven takes to warm up til 500ºC (in our case 1 hours on average).

7 Let the crucibles cool completely before removing the samples from the furnace.

Wet procedure

8

Note

These steps are fundamental to digest carbonates and oxidize organic material left from the ashing procedure and any form of organic material that was not removed.

Remove the ashes from the crucible with a spatula and place them in an Eppendorf tube of 5 ml. Use a clean spatula for each sample so not to contaminate between samples.

Ceramic crucibles containing the samples, covered with a lid. a) shows the plant tissue before ashing and b) shows the white ashes obtained after 12 hours in the oven.

9 Add 900 µL of 10% v/v HCl and vortex the tube to stir the solution (we use an ES714R Maxi Mixer). Leave the HCl to react for 05:00:00 (or till the reaction stops) 5h

with the cap of the tube open. Do not let the sample dry by adding more HCl solution if necessary.

Safety information

Work in a fume cupboard. Wear a lab coat and gloves when dealing with Hydrochloric acid.

10 Dilute the solution with 450 µL of distilled water added with a pipette and centrifuge the tubes at 6000 rpm for 00:05:00 (we use a Mini Spin Eppendorf®5453) after screwing on the lids of the centrifuge tubes. Discard the supernatant. Repeat this process for three more times to completely remove the HCl. 5m

11 Add 900 µL of H_2O_2(10% v/v). Place the tube in a dryer or alternatively in a hot bath at 40ºC until the reaction stops (in our experiments about 09:00:00 for chaff and leaves, at least 12 hours for stems) with the cap of the tube open. Every 2 hours rinse the samples with distilled water by repeating step 10 one time and add 900 µl H_2O_2. Do not let the sample dry by adding more H_2O_2 solution if necessary. 9h

Safety information

Work in a fume cupboard. Wear a lab coat and gloves when dealing with Hydrochloric acid

12 Dilute the solution with 450 µL of distilled water added with a pipette and centrifuge the tubes at 6000 rpm for 00:05:00 after screwing on the lids of the centrifuge tubes. Discard the supernatant. Repeat this process for three more times to completely remove the H_2O_2. 5m

13 Dry the extracts in a drying cabinet at 60ºC. When the powder is completely dry, cool the extracts and weigh them. Repeat the weighing until no weight loss is observed to be sure to measure the completely dry silica weight produced by the plant. Furthermore, when samples are dry the colour of the extract is usually clearer than when it is humid and the powder is loose (shake the tubes to check if the powder is loose or if lumps are still present, in this case there is still residual humidity).

Dry mounting

14 Place 0.0001 g of silica powder on a microscope slide with a clean spatula. Add 4 drops of Entellan New® on the top of the powder and gently stir using a micro clean spatula/micro spoon to distribute the phytoliths homogeneously. Cover the slide: we used a cover slip of 24 × 32 mm but also a 24 × 24 can be used. Use the same cover slip size for all slides.

Safety information

Wear gloves when dealing with Entellan New®

15 Leave the slide to dry for at least 30 minutes so that phytoliths set at the same depth. However, we suggest waiting about 12:00:00 so that Entellan New® reaches the best density (neither too liquid nor too dry) to rotate phytoliths to guarantee correct descriptions/identifications. 12h

Microscope observation and counting

16 Start counting and classify phytoliths. Always note the number of fields of view observed to calculate phytolith concentration later -it depends on how many fields of view are in one cover slip, indeed the measurement is related to the cover slip size. Rotate phytoliths to inspect their tridimensional shape and to assure a good classification of the morphotypes. Use the International Code for Phytolith Nomenclature (ICPN) 2.0 (Neumann et al. 2019) for the description of the morphotypes.

17

Note

Synonyms
silica skeletons: multi-cells structure
disarticulated cells: single celled, silica monoliths, single phytolith

Count until 50 skeletons have been numbered and their phytolith cells classified. For each skeleton, annotate the total number of cells for each morphotype recognized. While counting skeletons, count and classify all the disarticulated phytoliths along the pathway separately. At the end of the procedure, the information about phytoliths in skeletons and disarticulated cells can be summarized together in a unique file to obtain the total phytoliths size and the absolute number of each morphotype, as well as the silica skeleton sizes.