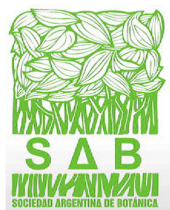

An easy technique for silicophytolith visualization in plants through tissue clearing and immersion oil mounting

Una técnica sencilla para la visualización de silicofitolitos en plantas mediante clarificación y montaje en aceite de inmersión

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Summary

Background and aims: Different methodologies were proposed for the detection of silica deposits in plant tissues. These methodologies include dry and wet ashing (which destroy the surrounding tissue), phenol staining (toxic), safranin–crystal violet lactone and fast green–methyl red staining (not specific for silica), fluorescence microscopy, electronic microscopy, EDAX and Raman analyses (which involve expensive equipment). Here we presented an easy and cheap method based on tissue clearing and immersion oil mounting.

M&M: We tested the methodology in longitudinal and cross sections of culms, leaves and roots of ten species that effectively accumulate silica. We applied different clearing techniques according to the type of plant material, we mounted in immersion oil and observed under light microscope. We compared the results with the ones obtained by traditional silicophytolith techniques.

Results: Silica deposits were observed in all species and organs analyzed, and the observations were coincident with the results obtained by other techniques. It was also possible to identify calcium crystals, allowing the description of the most common biomineralizations produced by plants.

Conclusions: The technique here proposed can be used for exploratory as well as for specific studies about the content and distribution of silicophytoliths in almost any tissue, organ and plant species. It can be applied in any laboratory, because it does not require expensive or hardly available equipment.

Key words

Anatomy, dicotyledons, *Equisetum*, immersion oil, monocotyledons, silicophytoliths, tissue clearing.

Resumen

Introducción y objetivos: Diversas metodologías han sido propuestas para la identificación de depósitos de sílice en los tejidos vegetales. Estas metodologías incluyen calcinaciones y digestiones químicas (destruyen el tejido que los contiene); tinción con fenol (tóxico); tinciones con safranina-cristal violeta y fast green-rojo metilo (no específicas para sílice); microscopía de fluorescencia, electrónica, EDAX y espectroscopía Raman (equipamiento costoso). En este trabajo se presenta un método económico y sencillo basado en la clarificación de los tejidos y su montaje en aceite de inmersión.

M&M: Testeamos el método en cortes longitudinales y transversales de hojas, tallos y raíces de diez especies que acumulan sílice. Aplicamos diferentes técnicas de clarificación de acuerdo al tipo de material, montamos en aceite de inmersión y observamos al microscopio óptico. Los resultados se compararon con los obtenidos por las técnicas tradicionales de silicofitolitos.

Resultados: Se observaron depósitos de sílice en todas las especies y órganos analizados, y éstos coinciden con los resultados obtenidos por las técnicas tradicionales. Asimismo, mediante esta técnica, fue posible identificar cristales de calcio, permitiendo la descripción de los dos tipos más comunes de biomineralizaciones en plantas.

Conclusiones: La técnica propuesta puede ser usada para estudios exploratorios, como así específicos, sobre el contenido y distribución de silicofitolitos en casi cualquier tipo de tejido, órganos y especie. Puede ser aplicado en cualquier laboratorio, debido a que no requiere de equipamiento costoso.

Palabras clave

Aceite de inmersión, anatomía, clarificación, dicotiledóneas, *Equisetum*, monocotiledóneas, silicofitolitos.

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Introduction

Diverse plant families accumulate amorphous silica ($SiO_2.nH_2O$) in intra or intercellular spaces of tissues, and these deposits, known as silicophytoliths or opal phytoliths, constitute up to 10% of the plant dry weight (Epstein, 1994; Hodson *et al.,* 2005; Exley, 2015). Silicon is taken up by roots from the soil solution in the form of monosilicic acid and is transported through xylem as mono- and disilicic acid (Ma *et al.*, 2002; Casey *et al.,* 2003). The uptake and movement of silicic acid is mediated by proteinaceous transporters and also by passive diffusion, depending on the species involved (Mitani & Ma, 2005; Ma *et al.* 2011; Exley, 2015). Regardless of the uptake system, silicic acid is translocated to the shoot via the xylem, where it is further concentrated, polymerized and deposited as amorphous silica (Ma & Takahashi, 2002; Exley, 2015).

Silicophytoliths have a botanical, anatomical and taxonomical relevance, since some of the morphologies produced are characteristic of specific taxa (Twiss, 1992; Prychid *et al.,* 2003; Piperno, 2006). Once the organ that contains the silicophytoliths is decomposed, these amorphous silica particles are incorporated to soils and sediments, where they can be preserved for thousands of years. Due to their taxonomical relevance and their good preservation in soils and sediments, they are widely used as indicators of past plant communities in paleontological and archaeological studies (e.g. Prasad *et al.*, 2005; Mercader *et al.*, 2010; Osterrieth *et al.*, 2016; Ball *et al.*, 2016). Moreover, silicophytoliths have an increasing interest on ecological and physiological researches, due to the roles reported for these deposits, such as anti-herbivore protection, abiotic stress and heavy metal amelioration, and light reflection (Richmond & Sussman, 2003; Ma, 2004; Cooke & Leishman, 2011).

The first step towards the comprehension of the silicification process in plants requires appropriate methodologies in order to characterize and identify the silica deposits in the tissues. Diverse authors have proposed different methodologies, and these include techniques that destroy or not the tissue containing the silicophytoliths (e.g. Johansen, 1940; Campos & Labouriau, 1969; Law & Exley, 2011). Dry ashing and wet ashing/acid extraction techniques remove or eliminate the organic matter through the burning of the plant material in a muffle furnace (dry ashing) or through the action of specific acids (wet ashing) (Campos & Labouriau, 1969; Parr *et al.,* 2001; Piperno, 2006; Jenkins, 2009). These techniques allow the isolation of silicophytoliths and their tridimensional observation, but do not permit the identification of the exact location of these deposits in tissues. Phenol, safranin–crystal violet lactone and fast green–methyl red staining techniques were used for the localization of silica deposits in tissues, however they are toxic or they have low specificity for silica (Johansen, 1940; Dayanandan *et al.,* 1983; Blecher *et al.,* 2012; Fernández Honaine & Osterrieth, 2012). Finally, the application of fluorescence microscopy, electronic microscopy and EDAX, low-voltage–high-contrast detection (vCD) and Raman analyses was shown to be successful for the identification of silicified cells, but they involve expensive equipment (Law & Exley, 2011; Blecher *et al.,* 2012; Soukup *et al.,* 2014; Dabney III *et al.*, 2016).

In the present study, an easy and low-cost technique for the detection of silicophytoliths in plant tissues is proposed, based on the methodology already used for phytolith analysis in soils and sediments. Silicophytoliths extracted from samples of soils and sediments are directly mounted in immersion oil or Canada Balsam, and then observed under optic microscope (e.g. Piperno, 2006; Osterrieth *et al.*, 2016). These mounting media have higher refractive indexes (1.54) than opal (1.42), and this fact allows a distinctly greater relief and a rose color of the silicophytoliths (Parry & Smithson,1957; Piperno, 2006). If a plant tissue fragment is well cleared and then it is mounted in immersion oil, it should be possible to distinguish the cells that have a silica deposit, both by the relief and by the rose color. This idea was also partially proposed by Parry & Smithson (1957, 1958), who found in botanical preparations that opal (silicophytoliths) became very conspicuous if the tissue surrounding them was colorless and sufficiently transparent. They applied different clearing techniques but only in leaves of grasses, and mounted them in cedarwood oil, Canada Balsam or Gurr´s neutral mounting media. They observed some silicified tissues, but they concluded that the best visualization of opals was get only

under polarizing or phase contrast microscopes (Parry & Smithson, 1958).

The present work aims to analyze the effectiveness of immersion oil mounting for the visualization of silicophytoliths *in situ* and under light microscope, in plant tissues previously cleared. For this purpose, leaves, stems and/or roots of ten species that effectively accumulate amorphous silica were selected for the study, and several types of histological and clearing techniques were applied. In order to analyze the effectiveness of the technique, the results were verified with those obtained by traditional methods used for silica detection, such as dry ashing (Campos & Labouriau, 1969), phenol staining and/or EDAX analyses.

Material and methods

Plant material

Leaves, stems and/or roots of ten species were sampled from natural areas of SE Buenos Aires province (Argentina) and from the Herbarium of Geoecología de Ambientes Sedimentarios laboratory, National University of Mar del Plata, Argentina (IGCyC, FCEyN, UNMdP-CIC) (Table 1). *Bothriochloa laguroides* seedlings were obtained from a laboratory assay, where seeds were germinated in river sand substrate with a solution of 1.8 mM Si and growth for 9 days in an environment-controlled chamber at a 16-h light/8-h dark regime, at 25 °C and 70% relative humidity (Fernández Honaine *et al.,* 2016). All the species selected produce silicophytoliths, which were already described by dry ashing techniques, phenol staining, scanning electron microscopy and EDAX (Zucol, 1999; Fernández Honaine *et al.,* 2009; 2016; 2017; 2018; Law & Exley, 2011; Fernández Honaine & Osterrieth, 2012; Benvenuto *et al.,* 2015; Benvenuto, 2017; De Rito *et al.,* 2018). The typical silicophytoliths produced by each species are detailed in Table 1.

Sectioning and clearing techniques applied

Free-hand cross and longitudinal sections were obtained from leaves and/or stems samples by standard methods (D'Ambrogio de Argüeso, 1986). For superficial viewing of the leaves, small pieces of the organs were selected and washed, previous to the application of the clearing technique. For root observation, the peripheral cortical tissues were mechanically removed with a razor blade, and so the stele covered by endodermal walls, where silica deposits occur, became exposed. Depending on the type and consistency of the material, samples were subjected to some of the following clearing techniques (Table 1):

- Clearing with sodium hypochlorite: samples were placed in small Petri dishes with sodium hypochlorite 50% until they turned transparent. Then, they were washed with distilled water until the sodium hypochlorite was removed.
- Dizeo de Strittmater clearing (D'Ambrogio de Argüeso, 1986): samples were placed in beakers with alcohol 96º and boiled in water bath for 5-10 minutes, depending on the consistency of the material. Then, the alcohol solution was replaced by a alcohol 96º:sodium hydroxide 5% (1:1) solution and boiled in water bath for another 5-10 minutes. The samples were washed with tap water and then, distilled water. Then, sodium hypochlorite 50% was added to the samples until they turned transparent. Finally, they were washed with abundant distilled water and stored in plastic bottles with chloral hydrate until they were mounted.
- Clearing with acetic acid and hydrogen peroxide (Motomura *et al.,* 2000): Samples were cleared with acetic acid and hydrogen peroxide (1:1) for 48 h at 60ºC. After that, samples were washed with distilled water and stored in alcohol 70% until they were mounted.
- Foster technique (D'Ambrogio de Argüeso, 1986): herbarium material was boiled in water for some minutes. Then, it was placed in Petri dishes with sodium hydroxide 5% at 50ºC for approximately 24 hs., until the material was clear. The sodium hydroxide solution was changed twice. After that, the material was placed in a beaker with distilled water for 24 hs., and then in chloral hydrate for at least one day.

Mounting media and observation under optic microscope

Cleared samples were mounted in slides and 1-2 drops of immersion oil were applied. Then, the samples were observed under Zeiss Axiostar Plus microscope at 400x magnification. Photographs were taken with a digital camera (Canon Powershot

Table 1. Species and organs selected for the study and the clearing technique applied.

Species (Family)	Site collection	Organ and sectioning	Clearing technique applied	Typical phytolith morphologies (references)
Celtis ehrenbergiana (Cannabaceae)	Herbarium from Geoecología de ambientes sedimentarios (IGCyC, FCEyN, UNMdP-CIC)	Leaf, superficial view	Dizeo de Strittmater clearing	Cystoliths, epidermal cells (Iriarte & Paz, 2009; Fernández Honaine *et al*., 2018)
Celtis occidentalis (Cannabaceae)	Herbarium from Geoecología de ambientes sedimentarios (IGCyC, FCEyN, UNMdP-CIC)	Leaf, superficial view	Dizeo de Strittmater clearing	Cystoliths, epidermal cells (Fernández Honaine *et al*., 2018; De Rito *et al*., 2018)
Ligustrum lucidum (Oleaceae)	Herbarium from Geoecología de ambientes sedimentarios (IGCyC, FCEyN, UNMdP-CIC)	Leaf, superficial view	Dizeo de Strittmater clearing	Tabular epidermical phytoliths (De Rito *et al*., 2018)
Phoenix canariensis (Arecaceae)	Fresh material, Mar del Plata city, Buenos Aires, Argentina	Leaf, free-hand cross-section	Sodium hypochlorite	Globular phytoliths (Tomlinson, 1961; Benvenuto *et al*., 2015)
		Leaf, superficial view	Acetic acid and hydrogen peroxide	
Trachycarpus fortunei (Arecaceae)	Fresh material, Mar del Plata city, Buenos Aires, Argentina	Leaf, free-hand cross-section	Sodium hypochlorite	Globular phytoliths (Tomlinson, 1961; Benvenuto *et al*., 2015)
Schoenoplectus californicus (Cyperaceae)	Fresh material, Mar Chiquita wetland, Buenos Aires, Argentina	Stem, free-hand cross-section	Sodium hypochlorite	Conical phytoliths, blocky phytoliths (Ollendorf, 1992; Fernández Honaine *et al*., 2009)
Bothriochloa laguroides (Poaceae)	Herbarium from Geoecología de ambientes sedimentarios (IGCyC, FCEyN, UNMdP-CIC)	Leaf, superficial view	Foster technique	Bilobates, bulliform phytoliths, elongate phytoliths (Fernández Honaine & Osterrieth, 2012)
		Root, longitudinal view	Sodium hypochlorite	Silica corpuscles in endodermis (Fernández Honaine *et al*., 2016)
Bothriochloa laguroides, seedling (Poaceae)	Fresh material	Entire plant, superficial view	Sodium hypochlorite	Bilobates (Fernández Honaine *et al*., 2016)
Cortaderia selloana (Poaceae)	Fresh material, Mar Chiquita wetland, Buenos Aires, Argentina	Leaf, superficial view	Acetic acid and hydrogen peroxide	Short silica cells, elongate phytoliths (Zucol, 1999; Fernández Honaine *et al*., 2017)
Triticum aestivum (Poaceae)	Herbarium from Geoecología de ambientes sedimentarios (IGCyC, FCEyN, UNMdP-CIC)	Leaf, superficial view	Acetic acid and hydrogen peroxide	Short silica cells, elongate phytoliths, stomata (Benvenuto, 2017)
Equisetum sp. (Equisetaceae)	Fresh material, Mar del Plata city, Buenos Aires, Argentina	Stem, free-hand cross-section and longitudinal section	Sodium hypochlorite	Stomata, epidermal cells (Law & Exley, 2011)

G10, Canon Inc., Tokyo, Japan). The observations were made in the same day and some days after the preparation of the slides, with the purpose to evaluate if the viewing of the sample was improved by time.

Some samples were dehydrated in an ethanol series up to 96%, before the mounting on immersion oil, in order to evaluate if this methodological step was relevant for a better visualization of the material.

Comparison with other silicophytolith detection and/or extraction techniques

With the purpose of analyzing the validity of the proposed technique, we compared the silica deposits observed in this study with the those described by the traditional methods for silicophytolith extraction or detection. These methods were: dry ashing method (Campos & Labouriau,1969), tissue clarification and phenol staining (Johansen, 1940; Fernández Honaine & Osterrieth, 2012) and SEM and EDAX analyses (JEOL JSM-6460 LV; Tokyo, Japan) at Universidad Nacional de Mar del Plata, Argentina. In this last case, samples were first fixed for 12 h with 3% glutaraldehyde, pH 7.2–7.4 phosphate buffered solution, followed by dehydration through an alcohol series (50, 70, 80, 90, 95 and 100%). Finally, samples were dried in HMDS (hexamethyldisilazane), mounted on aluminium discs and coated with gold. The composition of the silicophytoliths was analyzed using X-ray energy dispersive spectroscopy (EDS). The system used was an EDAX Genesis XM4-Sys 60, equipped with multichannel analyser EDAX mod EDAM IV, Sapphire Si (Li) detector and super ultra-thin window of Be, and EDAX Genesis version 5.11 software (Tokyo, Japan).

Results and Discussion

Depending on the type and consistency of the plant material, different clearing techniques were applied. In the three dicotyledon species studied here, the Dizeo de Stritmatter technique was the most adequate for this type of leaves; while in monocotyledons, acetic acid and hydrogen peroxide and Foster techniques were used for transparent the leaves (D'Ambrogio de Argüeso, 1986; Motomura *et al.*, 2000). Free-hand cross sectioned samples were all cleared with sodium hypochlorite 50%. Some sections were dehydrated in an alcohol series, but the results did not differ from those obtained from sections not dehydrated. The time of exposition in the immersion oil, i.e. the time between the sample is mounted until it is observed under microscope, seems to be a factor that improves the visualization of silica deposits. For instance, in the cross sections of palm leaves, the better results were obtained after three days.

In all the species and organs analyzed, the mounting in immersion oil of the cleared samples allowed the identification of the typical silicified cells produced in each species, according to previous studies (Table 1). Calcination technique showed that cystoliths and epidermical silica deposits are the dominant silicophytoliths produced in *Celtis* spp. leaves (Fig. 1 A, B) (Wallis, 2003; Iriarte & Paz, 2009; Fernández Honaine *et al.*, 2018; De Rito *et al.*, 2018). These silica deposits were easily identified in cleared tissues mounted in immersion oil. Also, calcium crystals, a very common biomineralization in this genus, were distinguished through this technique (Fig. 1C-E). Leaves of *Ligustrum lucidum* accumulate silica in epidermal cells, as it was observed through calcination techniques (Fig.1F) (De Rito *et al.*, 2018). These deposits were also identified with the technique proposed in this study (Fig. 1G).

Silicification process in monocotyledons, such as Poaceae, Cyperaceae and Arecaceae, is a common phenomenon. Short silica cells, bulliform cells, stomata and long cells are usually silicified in leaves of diverse species of grasses (Twiss *et al.*, 1992; Piperno, 2006). Figure 2 shows the silica deposits in short cells of leaves of *Cortaderia selloana* (Fig. 2A-B) and *Bothriochloa laguroides* (Fig. 2C-D), obtained through mapping analyses by SEM and EDAX procedures; and short silicified cells from leaves of *Triticum aestivum* obtained after a calcination technique (Fig. 2E) (Zucol, 1999; Fernández Honaine *et al.*, 2016; 2017; Benvenuto, 2017). Also, silicified bulliforms, stomata, short cells and bicellular hairs of leaves of *Bothriochloa laguroides* were detected through phenol staining technique (Fig. 2F) (Fernández Honaine & Osterrieth, 2012). All these silica deposits in the epidermis of leaves of grasses, previously detected by diverse methods (such as dry ashing, phenol staining and SEM/EDAX analyses), were clearly identified by the technique proposed in this study. Figure 2 showed the silica deposits (view in color) in epidermal cells of leaves of *Cortaderia*

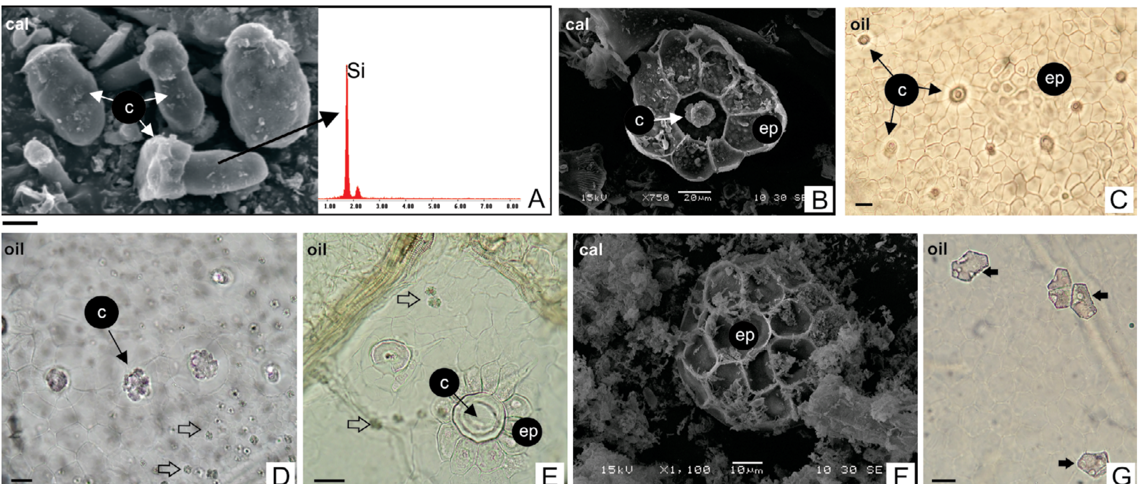

Fig. 1. Silicophytoliths in dicotyledons obtained through the calcination technique and their location in the tissue by the method proposed in this study. **A.** Photograph at SEM of silicified cystoliths obtained by a calcination technique in *Celtis ehrenbergiana*, and EDAX analyses of them. **B**. Above-view photograph at SEM of silicified epidermal cells and cystolith obtained by a calcination technique in *Celtis ehrenbergiana*. **C.** Silicified cystoliths and epidermal cells in cleared leaves of *C. ehrenbergiana* mounted on immersion oil. **D**. Silicified cystoliths and druses (calcium crystals) in cleared leaves of *C. ehrenbergiana* mounted on immersion oil. **E.** Calcium crystals (druses) and silicified cystoliths and epidermal cells in cleared leaves of *C. occidentalis* mounted on immersion oil. **F.** Photograph at SEM of silicified epidermal cells obtained by a calcination technique in leaves of *Ligustrum lucidum*. **G**. Silicified and not silicified epidermal cells in cleared leaves of *L. lucidum* mounted on immersion oil. Black arrow: silica deposits. Empty arrow: calcium crystals. Abbreviations= cal: calcination technique, oil: immersion oil technique (this study), c: cystoliths, ep: silicified epidermal cells. Scale bars= A: 10 µm, C, D: 20 µm, G: 25 µm.

selloana (Fig. 2G), *Bothriochloa laguroides* (Fig. 2H-I) and *Triticum aestivum* (Fig. 2J-K).

Conical silica deposits in epidermal cells associated to sclerenchyma tissue, and silica deposits in parenchyma cells are commonly present in leaves and stems of sedges (Fig. 2L-M) (Duval-Jouve, 1873; Ollendorf, 1992; Piperno, 2006, Fernández Honaine *et al.*, 2009). Cleared free-hand cross sections of stems of *Schoenoplectus californicus* mounted in immersion oil allowed the detection of these silica deposits, both in epidermal as in parenchyma cells (Fig. 2N-O).

Palm leaves accumulate high quantities of amorphous silica in their tissues, and the most abundant and typical phytolith morphologies are globular echinate deposits produced in parenchyma tissue (Fig. 3A-B) (Tomlinson, 1961; Kealhofer & Piperno, 1998; Iriarte & Paz, 2009; Benvenuto *et al.*, 2015). Cross and longitudinal sections of leaves of *Trachycarpus fortunei* and *Phoenix canariensis*, previous cleared and mounted in immersion oil, showed the location of these globular phytoliths in mesophyll and around vascular bundles (Fig. 3C-E).

Equisetum comprises a genus that accumulates high quantities of amorphous silica in the tissues (Hodson *et al.*, 2005; Law & Exley, 2011). Previous work based on fluorescence microscopy showed that silica is deposited around stomata complexes and in other epidermal cells (Law & Exley, 2011). Calcination techniques applied to stems to *Equisetum* sp showed some of the typical morphologies produced in this taxon (Fig. 3F-G) (Fernández Honaine et al., data not published). Cross and longitudinal sections of stems of *Equisetum* sp subjected to a clearing technique and mounted in immersion oil showed the location of the silica deposits in the tissues (Fig. 3H-J).

Roots also accumulate silicophytoliths, and grasses represent one of the main producers of root silica (Lux *et al.*, 2002, 2003). The roots of *Bothriochloa laguroides*, like other Andropogoneae grasses such as *Sorghum* sp., produce silica corpuscles associated to the internal wall of endodermis (Lux *et al.*, 2002; 2003; Fernández Honaine *et al.*, 2016). These silica corpuscles were observed by different methodologies like electronic microscopy and EDAX,

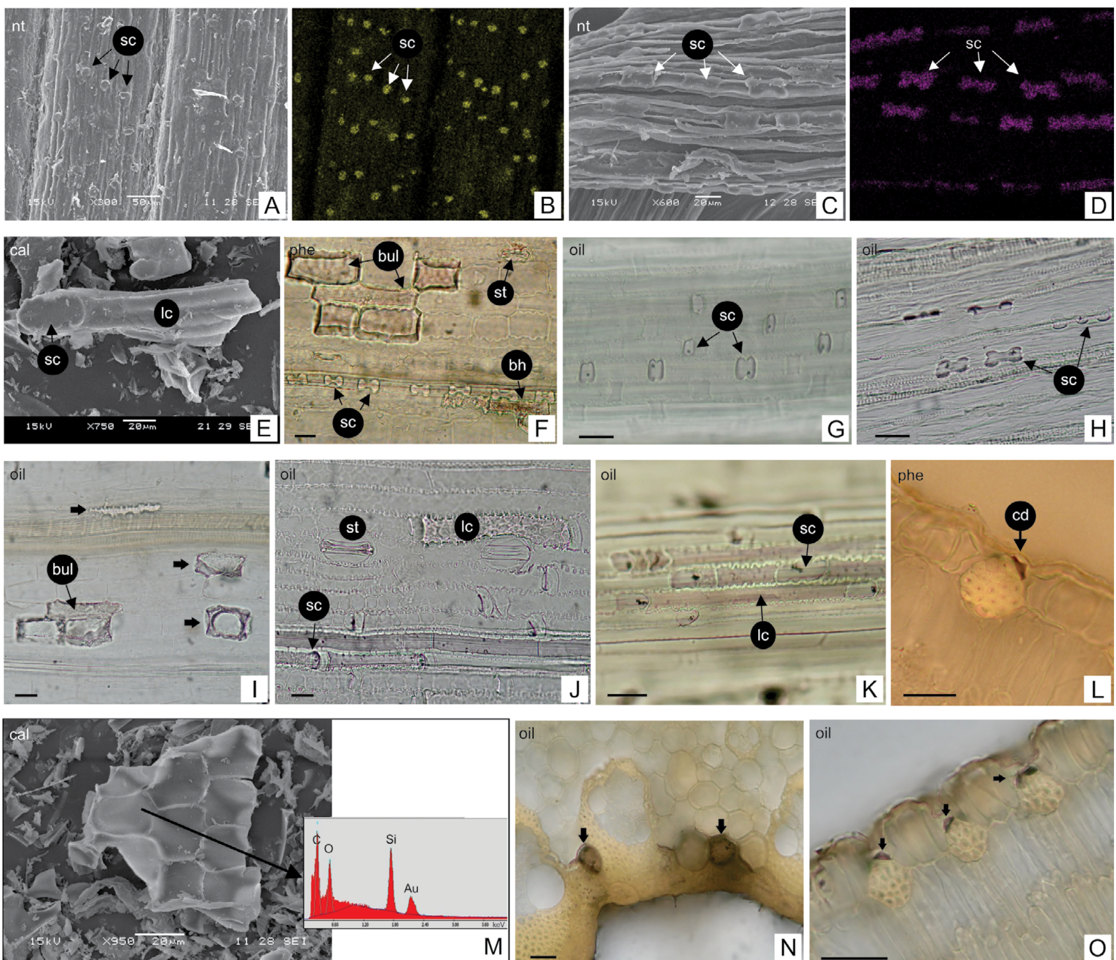

Fig. 2. Silicophytoliths in grasses and sedges. **A**. Photograph at SEM of adaxial epidermis of leaves of *Cortaderia selloana* showing location of short cells. **B**. Mapping of Si of the photograph in A, showing the silica deposit in short cells. **C**. Photograph at SEM of adaxial epidermis of leaves of *Bothriochloa laguroides* showing the location of short cells (bilobates). **D**. Mapping of Si of the photograph in C, showing the silica deposit in short cells (bilobates). **E.** Photograph at SEM of short and long silicified cells obtained by calcination technique in leaves of *Triticum aestivum*. **F**. Silicified cells (bulliform, stomata, short cells and bicellular hairs) in epidermis of leaves of *Bothriochloa laguroides* stained with phenol. **G**. Silicified short cells of epidermis of cleared leaves of *Cortaderia selloana* mounted on immersion oil. **H**. Silicified short cells of epidermis of cleared leaves of seedlings of *Bothriochloa laguroides* mounted on immersion oil. **I.** Silicified bulliform and epidermal long cells of cleared leaves of *Bothriochloa laguroides* mounted on immersion oil. **J**. Silicified epidermal long and short cells and stomata of cleared leaves of *Triticum aestivum* mounted on immersion oil. **K**. Silicified epidermal short and long cells of cleared leaves of *Triticum aestivum* mounted on immersion oil. **L.** Cross section of stem of *Schoenoplectus californicus* stained with phenol, showing the conical silica deposits in epidermis. **M**. Photograph at SEM of blocky silicophytoliths of stem of *Schoenoplectus californicus* obtained by a calcination technique, and the EDAX analyses associated. **N.** Cross section of stem of *Schoenoplectus californicus*, cleared and mounted on immersion oil, showing the silica deposits in parenchymatic cells. **O.** Cross section of stem of *Schoenoplectus californicus*, cleared and mounted on immersion oil, showing the conical silica deposits in epidermis. Black arrow: silica deposits. Abbreviations= nt: leaves without treatment, observed under SEM, phe: phenol staining, cal: calcination technique, oil: immersion oil technique (this study), sc: silicified short cells, lc: silicified long cells, bul: silicified bulliform cells, bh: silicified bicellular hairs, st: silicified stomata, cd: conical silica deposit. Scale bars= F, G, H, I, J, K, N, O: 25 µm, L: 20 µm.

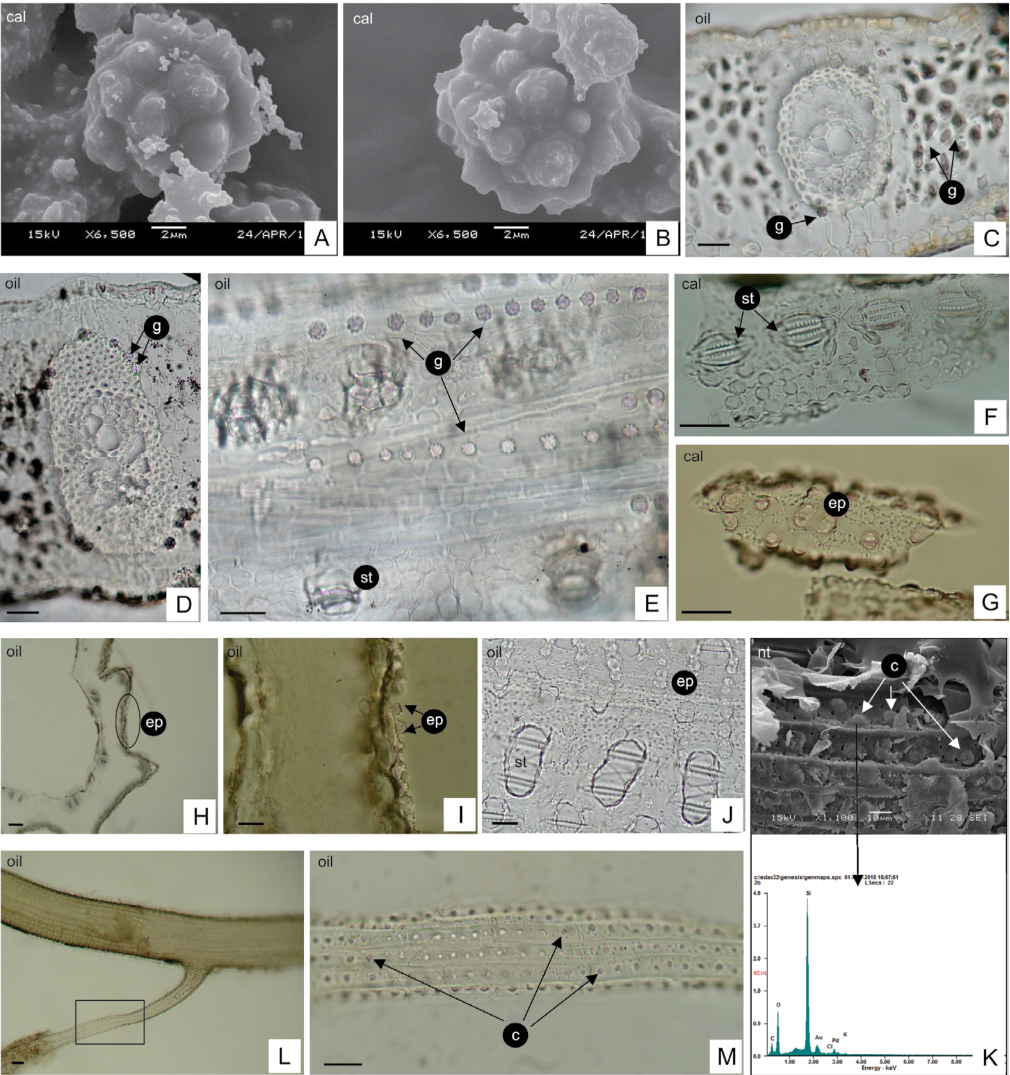

Fig. 3. Silicophytoliths in palms, *Equisetum* sp. and grass roots. **A, B**. Photographs at SEM of globular silicophytoliths obtained after calcination technique in leaves of *Trachycarpus fortunei* (**A**) and *Phoneix canariensis* (**B**). **C.** Cross section of cleared leaf of *Trachycarpus fortunei* mounted on immersion oil showing globular silica deposits around vascular bundle and in mesophyll. **D**. Cross section of cleared leaf of *Phoenix canariensis* mounted on immersion oil showing globular silica deposits around vascular bundle. **E**. Superficial view of cleared leaf of *Phoenix canariensis* mounted on immersion oil, showing the distribution of globular deposits and silicified stomata complexes. **F**. Silicified epidermis obtained through calcination technique in stems of *Equisetum* sp. **G**. Silicified stomata and epidermis obtained through calcination technique in stems of *Equisetum* sp. **H**. Cross section of cleared stem of *Equisetum* sp mounted on immersion oil, showing the location of silicified cells in epidermis (circles). **I**. Detailed of the area circle in H, showing the silica deposition in epidermal cells. **J**. Superficial view of cleared stems of *Equisetum* sp mounted on immersion oil, showing the silicified cells in epidermis and stomata complexes. **K**. Photograph at SEM of a longitudinal section of a root without cortex of *Bothriochloa laguroides* showing the silica corpuscles in the endodermis, and EDAX analyses of them. **L**. Panoramic view of principal and lateral root of *Bothriochloa laguroides* without cortex, cleared and mounted on immersion oil. **M**. Detailed of L, showing the corpuscles in endodermis. Abbreviations = nt: leaves without treatment, observed under SEM, cal: calcination technique, oil: immersion oil technique (this study), g: globular silica deposit, st: silicified stomata complexes, ep: silicified epidermal cells, c: silica corpuscles. Scale bars= C-G, I, J, M: 25 µm, H, L: 50 µm.

Table 2. Comparison of different techniques used for silicophytolith extraction or detection and the technique here proposed.

Techniques	Calcination technique (dry ashing)	Staining (phenol, green–methyl red or safranin–crystal violet lactone)	SEM-EDAX analyses	Fluorescence microscopy	Tissue clearing and immersion oil mounting
References	Campos & Labouriau (1969)	Johansen (1940), Dayanandan *et al*. (1983), Fernández Honaine & Osterrieth (2012)		Law & Exley (2011)	This study
Advantages	Simple preparation of the sample Medium to low cost of supplies Tridimensional description of silicophytolith morphologies	Simple preparation of the sample Medium to low cost of supplies Identification of silica and calcium deposits in tissue of origin	Identification of silica and calcium phytoliths in tissue of origin High specificity for silica Time consumed (from sample preparation to technique applications): 1-2 days approx.	Identification of silica phytoliths in tissue of origin High specificity for silica	Simple preparation of the sample Low cost of supplies Non-toxic reactives Identification of silica and calcium phytoliths in tissue of origin Specificity for amorphous silica
Disadvantages	Use of toxic reactives (acids) Destruction of surrounding tissue No *in situ* detection Time consumed (from sample preparation to technique applications): 3-5 days approx.	Use of toxic reactives(phenol) Low specificity for silica (green–methyl red or safranin–crystal violet lactone) Time consumed (from sample preparation to technique applications): 3-5 days approx.	Complex preparation of the sample Use of expensive supplies and equipment	Use of specific markers Use of expensive supplies and equipment Time consumed (from sample preparation to technique applications): several days (see reference)	Time consumed (from sample preparation to technique applications): 2-5 days

fluorescence and phenol staining (Fig. 3K) (Lux *et al.*, 2003; Soukup *et al.*, 2014, Fernández Honaine *et al.*, 2016). In the present study, it is shown that the clearing of root fragments without cortex and its mounting in immersion oil, allow the identification of the mentioned silica corpuscles in *Bothriochloa laguroides* (Fig. 3L).

As it was detailed in the introduction, different methods have been applied for silicophytolith extraction or detection in plant tissues (Piperno, 2006). Dry or wet ashing techniques do not allow the observation of the silicophytoliths *in situ*, since they destroy the surrounding tissue; however, they are usually applied when the aim of the study is to describe the silicophytolith morphologies in a tridimensional view (Piperno, 2006). Staining techniques (phenol, green–methyl red or safranin–crystal violet lactone) have been used for detecting amorphous silica deposits in tissues, but they have two important disadvantages: they involve toxic substances and they have low specificity for silica (Blecher *et al.*, 2012; Soukup *et al.*, 2014). Finally, SEM-EDAX analyses and fluorescence microscopy imply the use of expensive and not always available equipment and supplies, although they have the advantage of a high specificity for silica (Blecher *et al.*, 2012). The technique here proposed has some advantages over these traditional methodologies used for silicophytolith detection: it allows *in situ* visualization of silicophytoliths, it involves low cost and non-toxic supplies and equipment, and can be used in almost any laboratory (Table 2). Lastly, the present technique allows the simultaneously visualization of the two most common biomineralizations produced in plant tissues: amorphous silica deposits and calcium crystals. This advantage is only shared with SEM-EDAX analyses; however, in this last case, the sample area to be evaluated may be lower.

Conclusions

Considering the initial idea proposed by Parry and Smithson (1958) and the techniques used in soil phytolith studies, we presented a simply and rapid method for the visualization and identification of silica deposits in plant tissues, based on clearing and immersion oil mounting. Once the material is cleared and mounted in immersion oil, silica deposits became more visible and conspicuous, due to the different refractive indexes of opal and mounting media (immersion oil, in this case). Our results showed that this technique is appropriate for silicophytolith observation in diverse tissues, organs and species, and in different anatomical sections. It was demonstrated that the observations can be made by the standard light microscope, and not necessary by polarized or fluorescence microscopes. The comparison with other techniques demonstrates that the results are in coincidence with the those obtained previously, with the advantage that the proposed technique is easier and does not imply expensive equipment or toxic components. It can be used for exploratory studies as well as for specific studies of distribution of silicophytoliths and/or as a complement to other silicophytolith extraction techniques (such as dry or wet ashing). Finally, it is important to remark that this technique also enables the identification of calcium crystals, allowing the simultaneously description of two of the most common type of biomineralizations in plants (calcium and silica biomineralizations) (Franceschi and Nakata, 2005; Piperno, 2006).

Author´s contribution

MFH designed the work. MFH and MLB carried on the methodology and prepared the figures. MFH, MLB and MO wrote the manuscript.

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