Supplementary Material

How important is carbon sequestration in phytoliths within the soil?

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Supplementary materials and methods

CryoSEM-EDX

In order to analyse the chemical composition of *in situ* silica-rich structures, we performed CryoSEM-EDX analyses on a sedge species native from southwestern Australia (*Schoenus caespititius*). In the field (32° 1’10.24”S, 115°58’47.14”E), culm segments from three individuals were placed on an aluminium grooved pin with optimal cutting temperature compound and immediately plunge-frozen into liquid N, therefore immobilizing and preserving cellular ions (Kotula et al. 2021; Oi et al. 2022). Samples were stored in liquid N until required.

Perfectly flat, transverse surfaces of frozen-hydrated culm samples (n = 3) were prepared by stepwise cryo-planing (1 micron; 0.5 micron; 0.25 micron; 0.1 micron) with glass knives on a cryo-microtome (FC7, Leica Microsystems). Samples were transported directly from the cryo-microtome to the cryo-preparation system (ACE600, Leica Microsystems) in the transfer shuttle, where they were coated with 20 nm Cr, without sublimation. Coated samples were then transported under vacuum in the transfer shuttle to a field emission scanning electron microscope (JEOL JSM-IT800 HR) fitted with a cryo-stage (VCT500, Leica Microsystems) and an Oxford Ultim-Max170 X-ray detector interfaced to Oxford Instruments AZtec software. Samples were analysed at –155°C, 15 kV, and a 1 nA beam current (measured using a probe current detector in the column). The instrument was calibrated using a pure copper standard. Elemental maps were acquired at 512 pixel resolution for >2000 frames with a dwell time of 10 µs per pixel. Drift correction and pulse-pile up correction were activated. Three, four, or five maps were collected for each of the three replicates at different magnifications (from x190 to x2500), for a total of 12 maps taken for this species.

Quantitative data were extracted from regions of interest drawn on the 12 element maps, with individual spectra from each pixel summed and processed to yield concentration data using the Oxford Instruments AZtec software with inbuilt standards (Marshall 2017; Kotula et al. 2021; Oi et al. 2022). Three regions of interest were targetted: 1) conical-shaped phytoliths also called “cyperaceous type” and commonly found in epidermal cells of Cyperacea; 2) the outer tangential wall of the epidermis below the cuticle where Si was

detected; and 3) cell walls with no obvious Si deposition. Maps showing typical regions of interest analysed for this species are presented in Figure S1. Since this species does not show silica deposits in cell lumens (lumen phytoliths), we also analysed cross-sections of rice (sampled, prepared, and analysed in an identical manner) already available from another project. The region analysed from rice cross-sections is shown in Figure S4.

FIB preparation of TEM sample

Samples previously frozen in liquid nitrogen were dehydrated through a graded ethanol series and infiltrated with graded acetone : Hard Plus resin 812 mixtures using a BioWave Pro Microwave Processor (Ted Pella). Final embedding was performed using Hard Plus resin 812 in flat moulds at 60°C for 24 h. A transverse profile through the embedded sample was prepared using an ultramicrotome (UC6, Leica Microsystems), then the sample was mounted upright on an aluminium stub and coated with 20 nm Au. A thin section (<250 nm) of material for TEM was subsequently prepared from the middle of a single conical phytolith using FIB-SEM (Helios G3 CX, ThermoFisher Scientific) and this process is presented in Figure S2. First, a protective Pt strip is deposited on top of the region of interest (ROI), which in this case is the middle of a conical phytolith (Fig. S2a). Troughs are milled on each side of the ROI using the ion beam (30 kV; 0.79 nA beam current, 52 degree tilt) to remove excess material, resulting in a semi-thin (~1.5 micron-thick) section of sample precisely cut at the ROI (Fig. S2b-d). This semi-thin section is then cut from the main sample using the ion beam (Fig. S2e), attached to a micro-needle using a Pt weld, lifted out of the trench, and attached to a post on a Cu grid (PELCO FIB lift out TEM half grid) again using a Pt weld (Fig. S2f). Once mounted on a Cu post (Fig. S2g), the sample is progressively thinned using the ion beam (30 kV; 0.79 nA beam current, 51/53 degree tilt) until the desired thickness is achieved or the sample begins to bend (whichever comes first!) (Fig. S2h). A short, final cleaning step with the ion beam (5 kV; 0.41 nA beam current, 47/57 degree tilt) completes the process.

TEM Imaging and EDX

High Angle Annular Dark Field Scanning Transmission Electron Microscopy (HAADF-STEM) images and STEM-EDX maps were acquired at 200 kV using a TEM (JEOL F200

CF-HR) equipped with a JEOL 100 mm^2 silicon drift EDX detector controlled by Oxford Instruments AZtec software. The sample previously prepared with the FIB was carefully placed into a JEOL double-tilt Be low-background sample holder to minimise anomalous background signals during EDX analysis. HAADF-STEM images were acquired at a resolution of ~ 0.5 nm, with 3072 x 3072 pixels and a dwell time of 30 µs per pixel. The images are formed using electrons that are scattered to high angles by the sample, resulting in an image where the intensity is determined by the mass of the sample – with regions containing heavy elements, e.g. Si, appearing brighter that those containing light elements, e.g. C.

EDX elemental maps were acquired using the same probe size with the sample tilted ~18 degrees towards the detector to maximise signal. Maps were acquired from selected sub-areas of a 1024 x 1024 pixel reference image, with the final maps having 500-1000 pixels in each dimension. The maps used a dwell time of 100 µs per pixel, with each map being the sum of 40-50 repeat frames. Drift correction was activated. Quantitative numerical data were extracted from regions of interest drawn on the element maps (Figure S3), with individual spectra from each pixel summed and processed to yield concentration data using the Oxford Instruments AZtec software with inbuilt standards.

Supplementary figures

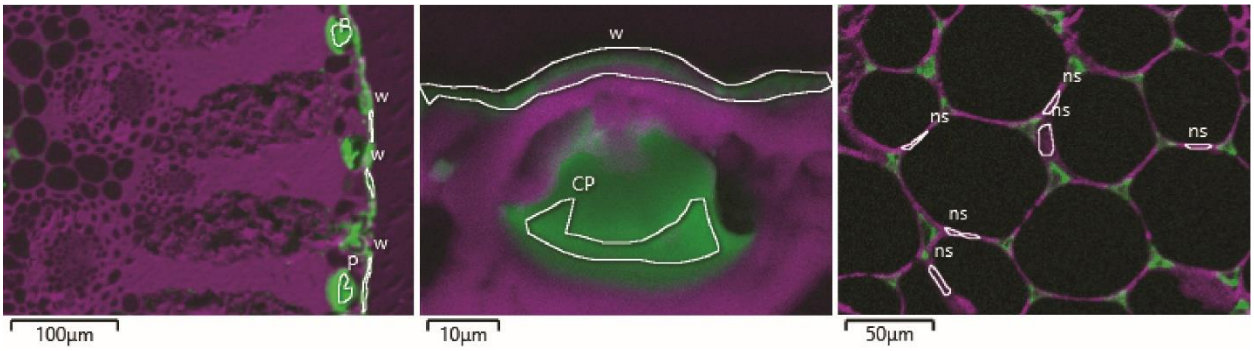

Figure S1 – Representation of the three different regions of interest analysed using cryoSEM-EDX in *Schoenus caespititius*: conical phytoliths (CP), Si-rich cell walls (w) and Si-free cell walls (ns). C is shown in purple, Si is shown in green. Scale bars: (a) 100 µm, (b) 10 µm, (c) 50 µm.

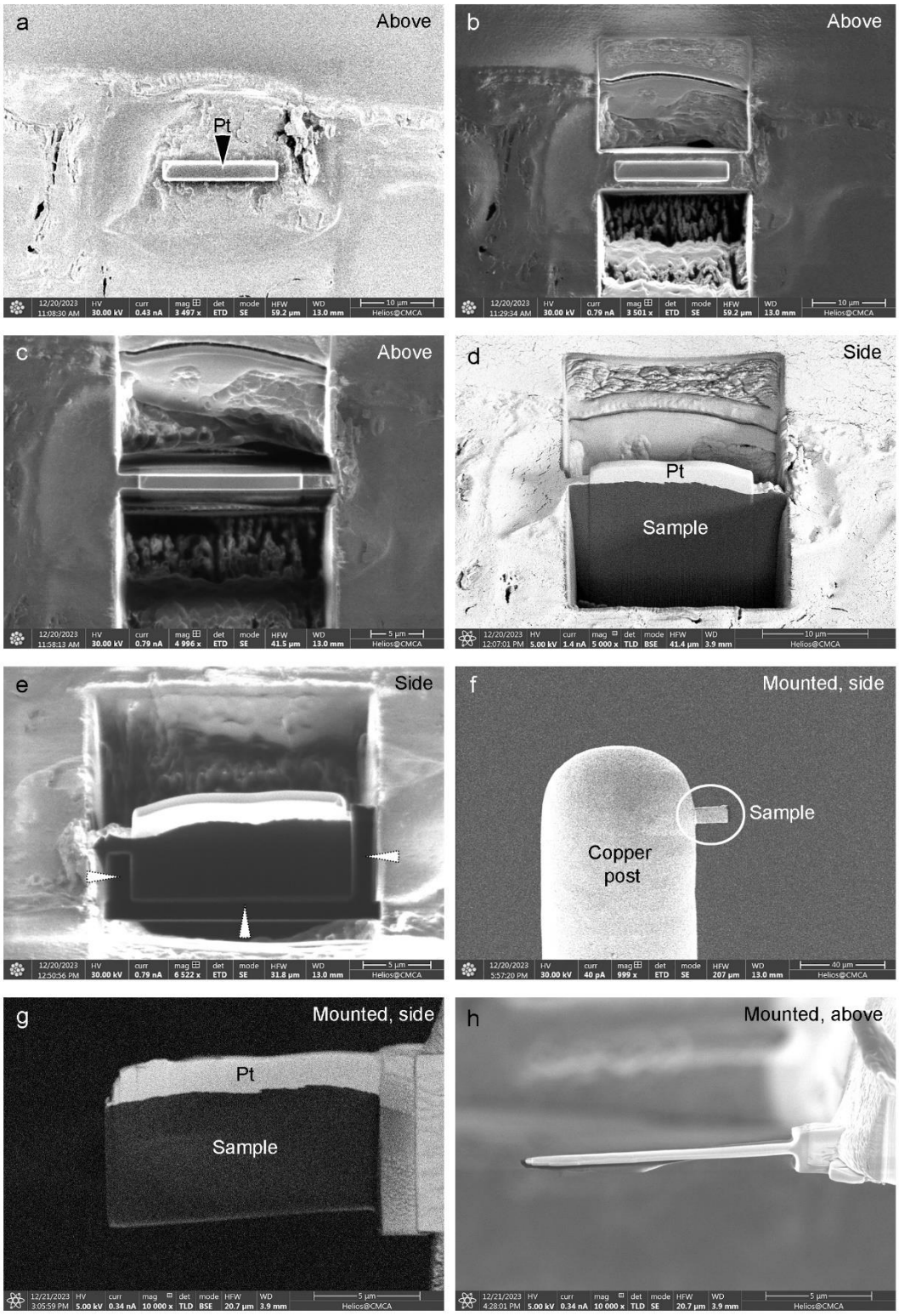

Figure S2 – Main steps involved in preparation of a TEM section from a conical phytolith using FIB-SEM, with views from above and from the side as needed. (a) A protective

platinum (Pt) strip is deposited on top of the region of interest (= centre of a conical phytolith); (b-d) trenches are then milled on each side to create a precisely located semi-thin section of sample; e) the section is then cut (white arrows) from the main sample; and (f-g) mounted on a Copper TEM grid before (h) being progressively thinned to the required thickness. Scale bars: 10 µm (a, b, d), 5 µm (c, e, g, h), 40 µm (f).

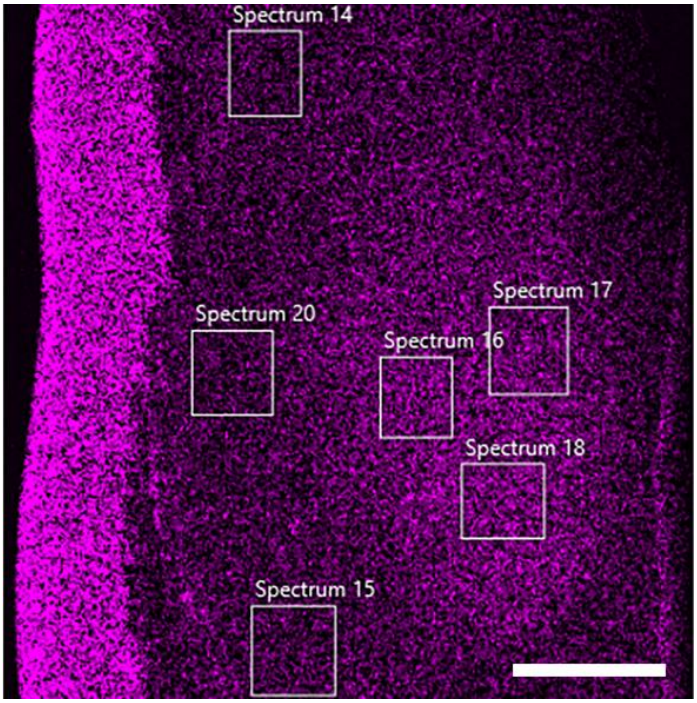

Figure S3: Representation of regions of interest drawn on the TEM-EDX maps to obtain C concentrations from the discreet C-rich core and surrounding areas of the FIB-prepared section. Scale bar: 2 µm.

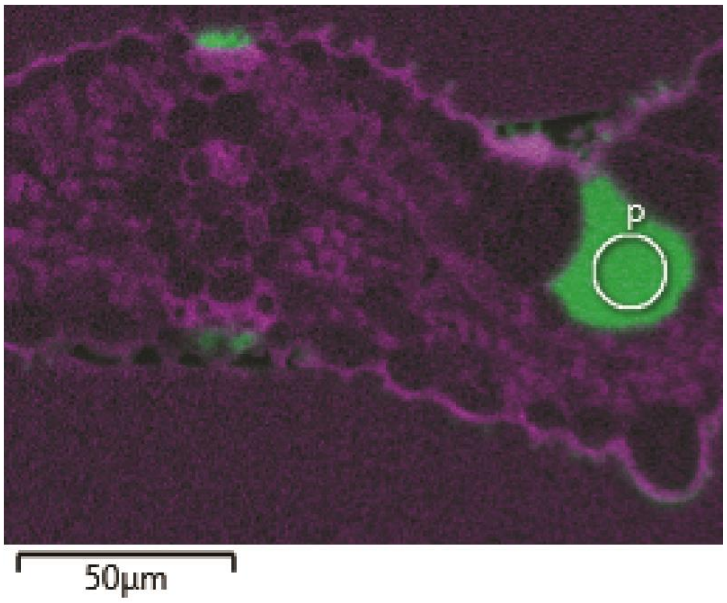

Figure S4: Representation of the region of interest analysed using cryoSEM-EDX in a rice lumen phytolith (p). C is shown in purple, Si is shown in green. Scale bar: 50 µm.

Supplementary results

In order to allow an easy and continuous improvement of PhytOC dynamics estimations, we share the Excel spreadsheet that allowed us to make calculations. We provide it in the online version of this article.

Supplementary references

Kotula L, Clode PL, Ranathunge K, Lambers H (2021) Role of roots in adaptation of soil-indifferent Proteaceae to calcareous soils in south-Western Australia. J Exp Bot 72:1490–1505. https://doi.org/10.1093/jxb/eraa515

Marshall AT (2017) Quantitative x-ray microanalysis of model biological samples in the SEM using remote standards and the XPP analytical model. J Microsc 266:231–238. https://doi.org/10.1111/jmi.12531

Oi T, Clode PL, Taniguchi M, et al (2022) Salt tolerance in relation to elemental concentrations in leaf cell vacuoles and chloroplasts of a C4 monocotyledonous halophyte. Plant Cell Environ 45:1490–1506. https://doi.org/10.1111/pce.14279