

New method for visualization of silica phytoliths in *Sorghum bicolor* roots by fluorescence microscopy revealed silicate concentration-dependent phytolith formation

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Abstract Silica phytoliths are microscopic structures of amorphous hydrated silica ($\text{SiO}_2 \cdot n\text{H}_2\text{O}$) formed by specialized plant cells. Besides their biological roles, physical, chemical, and structural properties of biogenic silica offer a wide spectrum of applications in many fields of industry and technology. Therefore, processes involved in their formation recently become a very interesting topic to study. However, optical transparency and microscopic sizes of silica phytoliths do not allow their visualization and localization by classical light microscopy methods. Their observation thus requires phytolith isolation, technically difficult or lengthy sample preparation procedures, or a work with toxic chemicals. In this paper we are proposing a novel method for visualization of silica phytoliths in *Sorghum bicolor* root endodermal cells by fluorescence microscopy using alkali mounting solution (pH 12). This method offers an easy and quick preparation of the samples and high contrast imaging. Based on our results we can assume that the proposed fluorescent method for silica

phytolith investigation allows observation of multiple samples in relatively short time period and thus might be applicable also for high-throughput screenings. Using this method we found out that after a 3-day cultivation of sorghum plants the minimal needed concentration of sodium silicate, limiting the formation of silica phytoliths in the root endodermis, was $25 \mu\text{mol dm}^{-3}$. The positive correlation of sodium silicate concentration in the substrate with the phytolith diameter was also observed.

Keywords Fluorescence microscopy · Phytoliths · Root endodermis · Silica · *Sorghum bicolor*

Introduction

Biomineralization is the formation of inorganic materials in biological systems. Development of such structures is driven by organic templates that allow to design a great variety of specific shapes and compositions (Weiner and Dove 2003; Gröger et al. 2008; Gong et al. 2010; He et al. 2014). Silicon is one of the most common elements involved in biomineralization processes by forming an amorphous hydrated silica minerals ($\text{SiO}_2 \cdot n\text{H}_2\text{O}$), that are the main components of diatomic thecae, spicules of sponges, and plant silica phytoliths (Currie and Perry 2007; Bauer et al. 2011; Law and Exley 2011; He et al. 2014). Besides their biological roles, silica materials possess many structural, physical and chemical features applicable also in biomedicine, nanotechnology, electronics, construction industry, and others (Sumper and Kröger 2004; Sun et al. 2004; Gröger et al. 2009; Neethirajan et al. 2009). However, their artificial preparation requires quite difficult technological conditions such as an extreme pH and temperature, and does not provide sufficient ultrastructural and

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morphological variability found in living organisms (Sun et al. 2004; Foo et al. 2004; Belton et al. 2008; Neethirajan et al. 2009; Gong et al. 2010). Therefore, new insights into biological processes involved in silica biomineralization might offer considerable advances in many fields of industry.

Orthosilicic acid, present in almost all types of soils, represents the main source of silicon for biological systems (Cornelis et al. 2011; Balakhnina et al. 2012; Song et al. 2012). Many land plant species, especially horsetails and grasses, are able to accumulate orthosilicic acid actively from the substrate or a soil solution (Hodson et al. 2005; Law and Exley 2011) and utilise part of the available orthosilicic acid to form microscopic biominerals called silica phytoliths (silica aggregates or silica bodies) (Richmond and Sussman 2003; Wüst and Bustin 2003; Currie and Perry 2007; Neethirajan et al. 2009; Song et al. 2012). Besides improving mechanical properties of plant organs, these structures also provide several defence mechanisms against herbivores and pathogens. Moreover, their features help to protect against UV radiation, drought, mitigate toxic effects of some elements, e.g. Al, Cd, Zn, K, Na, Mn, etc. (Hattori et al. 2003; Lux et al. 2003; Wüst and Bustin 2003; Currie and Perry 2007; Neethirajan et al. 2009; Vaculík et al. 2009; Bauer et al. 2011; He et al. 2014; Klančnik et al. 2014). Phytoliths might achieve miscellaneous shapes such as dumb-belled, lobate, dendriform, rods, etc. with sizes ranging up to 200 μm (Lu and Liu 2003; Piperno 2006; Neethirajan et al. 2009). Many plant species might dispose with several different phytolith morphotypes, as well as there is a great morphological variability observed among the species and families (Lu and Liu 2003; Sun et al. 2004; Piperno 2006). Taxonomic specificity supported by their durable structure additionally designates their utilization as a suitable marker in palaeobotany and archaeology (Fernández-Honaine et al. 2006; Piperno 2006; Neethirajan et al. 2009; Bauer et al. 2011; Cabanes et al. 2011).

Besides phytoliths localised in aboveground organs, plants of *Sorghum bicolor* (L.) Moench form also large amounts of dome-shaped silica phytoliths, regularly arranged within the inner tangential wall of root endodermal cells (Sangster and Parry 1975; Lux et al. 2003). *Sorghum* thus might represent an adequate model organism for silica biomineralization studies in plants. However, microscopic size and optical transparency of silica phytoliths complicate observations of their morphology and distribution pattern in many plant tissues. Even though, there are several techniques used for visualization of silica phytoliths by various microscopy types, their utilization often require time-consuming sample preparation procedures, manipulation with carcinogenic chemicals, isolation of phytoliths from tissues, expensive observation charges

or hardly available equipment (Johansen 1940; Dayanandan et al. 1983; Blecher et al. 2012). These difficulties remarkably restrict application of high-throughput screenings necessary for biomineralization studies at cellular and subcellular level. In this work we propose a novel technique for silica phytolith visualization within the plant tissues by fluorescence microscopy that should facilitate further investigation on silica biomineralization in plants. Additionally, we compare the suggested fluorescent method with several recent light microscopy methods.

Materials and methods

Plant material and cultivation

Grains of *Sorghum bicolor* (L.) Moench, cv. Gadambalia, were sterilized in sodium hypochlorite (2.5 %) for 15 min and washed in distilled water. After 24 h imbibition in distilled water the grains were placed into a wet paper rolls and germinated for 72 h in cultivation chamber in dark, temperature 25 °C, and 60 % humidity. Seedlings were grown in hydroponic solutions composed either of distilled water only (Si 0) or distilled water supplied by various concentrations of sodium silicate (Na_2SiO_3) described below. The pH of solutions was adjusted to 5.8. Cultivation lasted for 72 h under controlled conditions with 16/8 h light/dark photoperiod with illumination of photosynthetic active radiation of approx. 125 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 25 °C/18 °C light/dark temperature, and 50 % air humidity. Samples from the basal region (20–30 mm from the root-shoot junction) of seminal roots were collected and processed specifically depending on the type of observation method. Root segment preparation for some observations required the rhizodermis and cortical tissues to be mechanically removed (Lux et al. 2003). Investigated peeled root thus consisted of stele covered by centripetal part of endodermis (tangential cell walls facing the pericycle and parts of anticlinal cell walls of endodermal cells). This technique exposes the inner surface of tangential cell walls of endodermis for direct observation.

To determine the critical concentration of sodium silicate in the substrate allowing formation of silica phytoliths in sorghum root endodermis and the relationship between the concentration gradient and phytolith sizes, we tested concentrations of 0.25, 2.5, 10, 25, 50, 100, 250, 1,000 and 2,500 $\mu\text{mol dm}^{-3}$ (variants Si 0.25; Si 2.5; Si 10; Si 25; Si 50; Si 100; Si 250, Si 1000, and Si 2500, respectively).

Fluorescence microscopy

The hand cross-sections and the peeled root segments from the basal part of seminal roots were used for observations

by fluorescence microscopy. Mounting solution for fluorescence microscopy consisted of distilled water with pH adjusted by NaOH and HCl (pH = 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0). The exact pH value during the observation could slightly fluctuate as a consequence of the presence of samples. Fluorescence microscopy was performed by fluorescence microscope Axioskop 2 Plus (Carl Zeiss) with filter set No. 2 (excitation filter TBP 400 + 495 + 570 nm, beamsplitter TFT 410 + 505 + 585 nm, and emission filter TBP 460 + 530 + 610 nm).

Spectrofluorimetric analysis

Samples for spectrofluorimetric analysis were prepared from sorghum plants cultivated in hydroponics with or without addition of Na₂SiO₃. The primary seminal root segments were taken from the 1–6 cm region from the root base. Rhizodermis, cortical tissues and lateral roots were mechanically removed, the samples were frozen by liquid nitrogen and homogenised with pestle and mortar. Material was placed into 1 cm quartz cuvettes filled with mounting solution with either pH 5 or pH 12. Samples were analysed by spectrofluorimeter FSP 920 (Edinburgh Instruments, UK) in the right-angle arrangement, using the temperature-controlled cuvette holder with excitation at wavelengths of 360, 400, and 430 nm.

Light microscopy

The peeled segments from the basal part of seminal roots were used for observations by light microscopy. Mounting solution for bright field (BF) and differential interference contrast (DIC) observations consisted either of distilled water only or contrast adjustment of silica phytoliths for bright field observation was performed by the phenol staining procedure (PS) according to Johansen (1940). Phenol crystal was placed on the sample and the slide was heated until the phenol crystal dissolved. The samples were observed by microscope Axioskop 2 Plus (Carl Zeiss).

Phytolith dissolution rate

To find out a dissolution potential of strong alkali mounting solution containing NaOH we compared the size differences of phytoliths during 60 min after the application of the mounting solution. Peeled root segments were placed on glass slides into mounting solution of pH 12, applied for fluorescence visualization described above. Phytolith diameters were subsequently measured every 180 s with either permanent UV-irradiation or UV-irradiation only during picture taking.

Digital image analysis

Morphological characteristics and fluorescence intensity of silica phytoliths were compared after digital documentation (digital camera DP72, Olympus) by image analysis software Lucia (v. 4.80, 2002, Laboratory Imaging, Prague, Czech Republic). Relative fluorescence intensity measurement was derived from the maximum measured intensity (at pH 12) that was set as 1.

Statistical analysis

In each treatment, at least 2500 silica phytoliths from 40 images taken from ten seminal roots for each treatment were used for analysis. Data are presented as the mean \pm s.e. and they were analysed by one-way analysis of variance (ANOVA, LSD test; Statgraphics Centurion XV, v. 15.2.05, StatPoint, Inc.).

Results and discussion

Visualization of silica phytoliths in sorghum root endodermis

The aim of this study was to contrive a new, easy and fast method for visualization of silica phytoliths in *S. bicolor* by fluorescence microscopy. Our main objective was the possibility of application of the suggested fluorescence method for experiments with multiple treatments or high-throughput screenings that could facilitate further studies on biomineralization in plants.

Fluorescence microscopy

We investigated the inducible autofluorescence of silica phytoliths in the endodermis of *S. bicolor* roots by high pH value mounting solutions. Fluorescence microscopy thus subsequently allows visualization of silica phytoliths in situ. Fluorescent signal emitted by silica phytoliths in distilled water (pH 5–7) does not achieve sufficient intensity and requires remarkable increase of the exposure time to be detected. On the root transversal sections a weak blue autofluorescence of cell walls, mostly in lignified tissues, was notable. Endodermal phytoliths emitted also a blue fluorescence that was weak and hardly distinguishable from cell walls. Observation of silica phytolith fluorescence in mounting solution of pH range 5–10 is thus not adequate for their determination in plant tissues (Figs. 1, 2).

Emission of blue fluorescent signal by silica phytoliths is remarkably enhanced in mounting solution with pH adjusted to 12. Observation of the root cross-sections

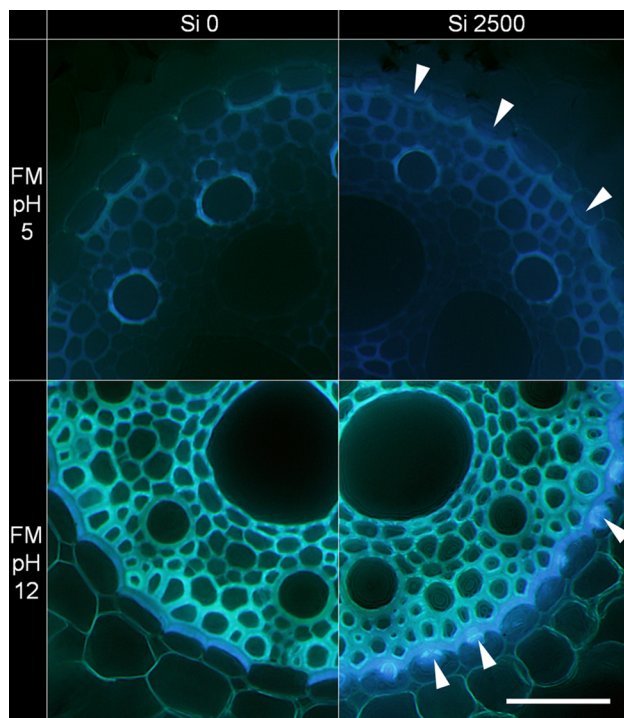


Fig. 1 Details of cross sections of *S. bicolor* primary seminal roots mounted in solutions of pH 5 (FM pH 5) or pH 12 (FM pH 12) visualized under fluorescence microscope. The plants were cultivated 3 days in substrates without (Si 0) and with addition of sodium silicate in final concentration of $2,500 \mu\text{mol dm}^{-3}$ (Si 2500). Silica phytoliths (arrows), mounting solutions of pH 5 and pH 12, scale bar $50 \mu\text{m}$

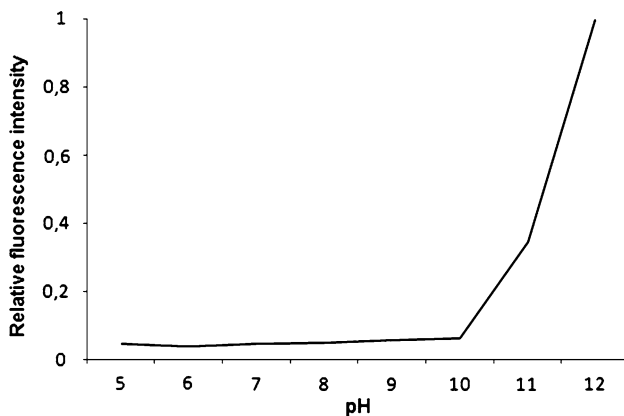


Fig. 2 Effect of mounting solution pH on the relative fluorescence intensity of silica phytoliths from root endodermal cells of *S. bicolor* plants cultivated in substrate with $2,500 \mu\text{mol dm}^{-3}$ final concentration of sodium silicate and observed under fluorescence microscope

showed phytolith localisation in cells and provided sufficient contrast for their microscopic morphological analysis. Comparison of treatments, with (Si 2500) and without (Si 0) sodium silicate in mounting solution with pH values adjusted to 5 and 12 are shown in Fig. 1.

Visualization of silica structures in fluorescence microscopy can be performed also by 2-(4-pyridyl)-5-((4-(2-dimethylaminoethylaminocarbonyl)methoxy)phenyl)oxazole (PDMPO) (Law and Exley 2011). However, that expects the addition of relatively expensive dye into the substrate of plants and cultivation with dye, which might complicate application of other requested treatments.

Effect of pH and time on phytolith fluorescence intensity

Phytolith fluorescence was nearly undetectable in wide range of mounting solution pH values (Fig. 2). From pH 5 up to pH 10, the detection of phytolith fluorescent signal

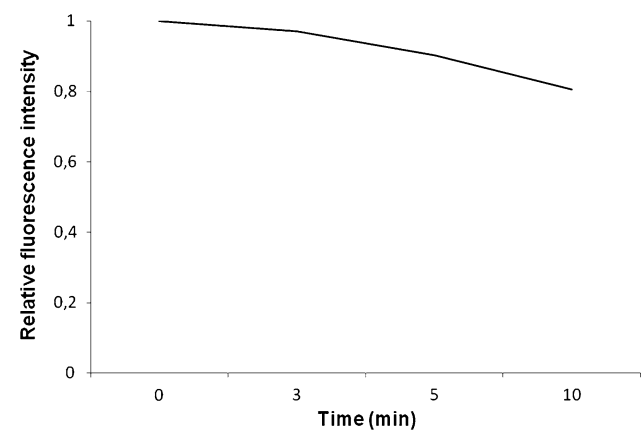


Fig. 3 Time dependent decrease of silica phytolith relative fluorescence intensity in pH 12 mounting solution observed under fluorescent microscope during continual UV-light irradiation. Plants were cultivated in substrate with addition of sodium silicate in final concentration of $2,500 \mu\text{mol dm}^{-3}$ (Si 2500)

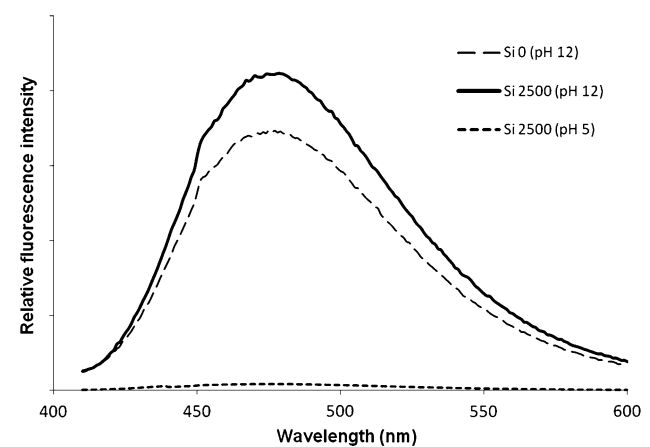
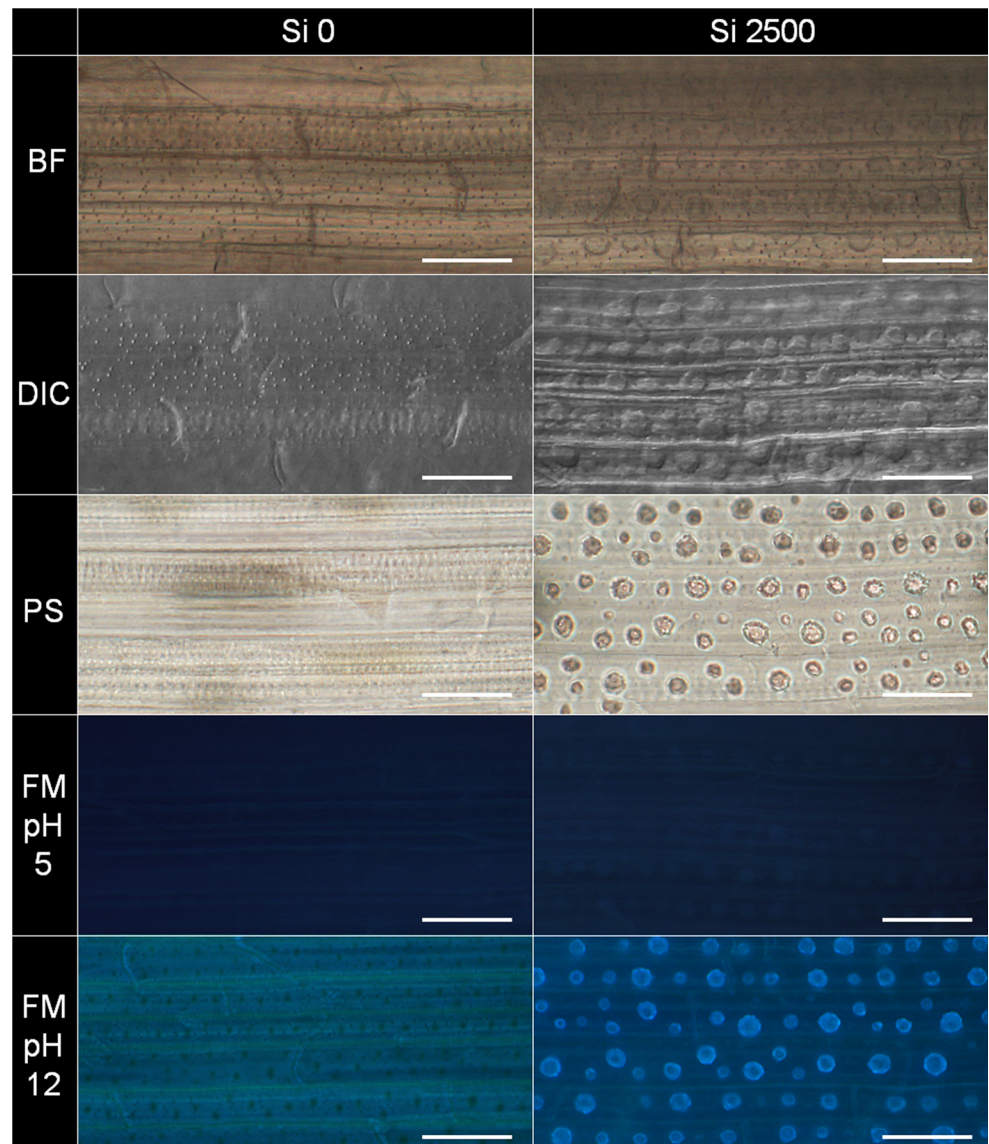


Fig. 4 Fluorescence emission spectra of homogenised root segments merged in mounting solution of pH 5 and pH 12. Excitation by light of 400 nm wavelength. Plants were cultivated in substrate without (Si 0) and with addition of sodium silicate in final concentration of $2,500 \mu\text{mol dm}^{-3}$ (Si 2500)

Fig. 5 Comparison of silica phytolith visualization techniques in root endodermal cells of *S. bicolor* plants cultivated in hydroponics without (Si 0) or with addition of sodium silicate in final concentration of $2,500 \mu\text{mol dm}^{-3}$ (Si 2500). Bright field (BF), differential interference contrast (DIC), phenol staining (PS), fluorescence microscopy with mounting solutions of pH 5 (FM pH 5) and pH 12 (FM pH 12). Scale bar 50 μm



was either not possible or required adequate imaging technologies like prolonged exposure time or highly sensitive camera sensor. Within these scales of pH, the autofluorescence of lignified tissues was also very high and might be misleading. Relative fluorescence intensity of silica phytoliths is almost without change within the pH values from 5 to 10 and varies between 4.04 and 6.26 %. Increased mounting solution pH above 10 remarkably enhanced the intensity of silica phytolith fluorescence. In pH 11 the relative fluorescence intensity increases to 34.71 %, and in pH 12 reaches the maximum within the tested pH range of mounting solutions.

Phytolith fluorescence induced by high pH values possesses relatively high stability character. Intensity of the fluorescence signal declines very slowly. Ten minutes after application of mounting solution (pH 12) and irradiation by UV-light the relative intensity decreased to 80.62 % of the

starting value (Fig. 3). The potential effect of alkali mounting solution on phytolith dissolution was tested during the period of 60 min with either continual UV-irradiation or with UV-irradiation only during capturing of the samples. No measurable phytolith dissolution was recorded in both observations.

Spectral analysis

Fluorescence emission spectra of segments from the both Si 2500 and Si 0 treatments excited by light of 400 nm wavelength reach the highest intensity with the maximum at 475–480 nm (according to Fig. 4). Comparing the emission spectra of peeled root segments of plants from Si 2500 and Si 0 treatments at pH 12 indicated that there was no significant difference in spectral composition of the emitted fluorescence. Excitation by other light wavelengths

(360 or 430 nm) did not show remarkable differences in the spectra composition (data not shown). Spectrofluorimetric analysis thus is not specific enough for quantification of silica phytoliths in tissues.

Light microscopy

Optical transparency and microscopic scales of silica phytoliths do not allow their proper identification within the plant tissues by light microscopy without any additional techniques or staining methods. Bright field observation (BF) does not provide sufficient contrast and thus is not suitable for phytolith detection or morphological studies (Fig. 5). Bright field observation of silica structures in plant tissues can be improved by application of phenol staining (PS) procedure, according to Johansen (1940). Phenol dissolution induces pale red staining of silica structures, what has been successfully used to visualize silica phytoliths of several species (Prychid et al. 2004; Benvenuto et al. 2013; Fernández-Honaine et al. 2013). Moreover, the phenol causes bleaching of surrounding tissues that increases the observation possibilities. Application of this method helps differing phytoliths from cell organelles, but often encountered complications such as phenol crystallization, its unequal distribution, or excessive formation of gas bubbles. Additionally, the main disadvantage is that toxic phenol vapour might be inhaled during the sample heating and cooling down.

Visualization of silica structures in light microscopy can be performed also by silver ammonium chromate, by methyl red or by crystal violet lactone as described in Dayanandan et al. (1983). However, remarkable unspecificity of application on intact plant material was reported in Blecher et al. (2012). Otherwise these methods require isolation of phytoliths from tissues and thus might not be used for their distribution studies in intact samples.

Application of differential interference contrast (DIC) offered better conditions for observation of silica phytoliths without any additional staining than the bright field (Fig. 5). Even though, the phytoliths can be often hardly distinguished from cell organelles and in case of abnormal distribution, their cellular localisation could not be exactly determined. Additionally, during observation by DIC the phytoliths remain covered by residues of endodermal plasmalemma that misguides information about their real dimensions and morphology.

Effect of sodium silicate concentration on phytolith proportions

Minimal diameter of sorghum root silica phytoliths detected by the suggested fluorescence microscopy visualization method was 1.36 μm observed in Si 25 variant and seemed

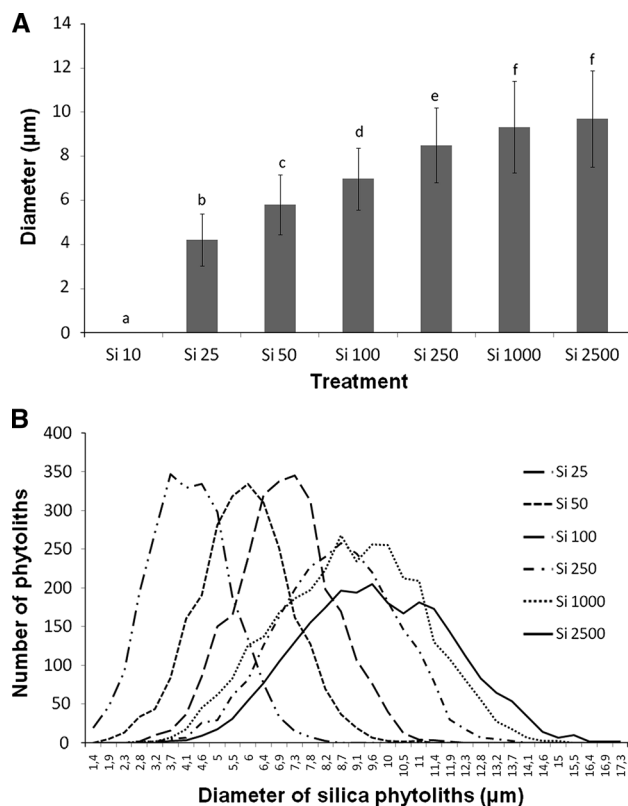


Fig. 6 Average diameters of silica phytoliths (a) and their frequency distribution (b) detected by fluorescence microscopy formed in root endodermal cells of sorghum plants cultivated in hydroponic treatments containing sodium silicate in increasing concentration. Concentrations shown are 10 $\mu\text{mol dm}^{-3}$ (Si 10), 25 $\mu\text{mol dm}^{-3}$ (Si 25), 50 $\mu\text{mol dm}^{-3}$ (Si 50), 100 $\mu\text{mol dm}^{-3}$ (Si 100), 250 $\mu\text{mol dm}^{-3}$ (Si 250), 1,000 $\mu\text{mol dm}^{-3}$ (Si 1000) and 2,500 $\mu\text{mol dm}^{-3}$ (Si 2500). Different letters above the columns represent statistically significant differences between the treatments; $n > 2,500$; mean \pm s.e.; ANOVA, LSD test; $P < 0.05$

to be limited only by the fluorescent microscope resolution possibilities. Concentration of 25 $\mu\text{mol dm}^{-3}$ of sodium silicate appeared to be limiting for the formation of phytoliths after a three day cultivation of experimental plants. Presence of phytoliths within the concentration range of 0.25–10 $\mu\text{mol dm}^{-3}$ of sodium silicate in the substrate was not recorded (data not shown). Average diameters of silica phytoliths and the frequency distribution of phytolith diameters suggested positive correlation with sodium silicate concentration in the substrate (Fig. 6). However, the concentration of 2,500 $\mu\text{mol dm}^{-3}$ did not cause significant increase in phytolith diameter comparing to 1,000 $\mu\text{mol dm}^{-3}$. According to Gröger et al. (2009) this might be caused by partial autopolymerization of silicic acid in the solution after exceeding the concentration of 2,000 $\mu\text{mol dm}^{-3}$. Number of phytoliths per endodermal cell after a three-day cultivation of plants in observed treatments counted from 4 to 18 (median = 10) with regular arrangement in one or two rows along the cell axis

with no significant difference between various concentrations of sodium silicate in the solution.

Conclusions

Due to optical features of silica phytoliths, their observation in light microscopy needed to be adjusted by silica specific stains. However, these methods require isolation of phytoliths from the tissues, work with highly toxic and carcinogenic phenol, or do not provide sufficient specificity. Visualization of silica phytoliths by fluorescence microscopy at high pH values described in this contribution is offering easy, fast, and cheap method for their basic morphological and distribution studies. This method allowed observation of multiple treatments in short time period and revealed the correlation between the sodium silicate concentration and the diameter of silica phytoliths in endodermal cells of sorghum primary roots. Based on our results from the used 3-day cultivation setup the minimal concentration limiting the formation of silica phytoliths in the root endodermis is $25 \mu\text{mol dm}^{-3}$ of sodium silicate. The suggested method might be applicable also for high-throughput screenings that should facilitate silica biomineralization studies in plants at the molecular level by tracking changes of phytolith formation caused by various external factors, or by identification of mutant plants.

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