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ORIGINAL PAPER

Development of the polysaccharidic matrix in biocrusts induced by a cyanobacterium inoculated in sand microcosms

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Abstract Soil inoculation with cyanobacteria (cyanobacterization) is a biotechnological method widely studied to improve soil quality and productivity. During their growth on soil, cyanobacteria excrete exopolysaccharides (EPSs) which glue trichomes to soil particles, in a three-dimensional extracellular polymeric matrix. EPS productivity is an important screening parameter to select proficient inoculants and is affected by growth conditions and abiotic stresses. In this study, we evaluated the capability of the cyanobacterium *Schizothrix* cf. *delicatissima* AMPL0116 to form biocrusts when inoculated in sand microcosms under stressing conditions, and the characteristics of the synthesized polymeric matrix. In parallel, we evaluated the characteristics of exopolysaccharidic exudates of the strain when grown in liquid culture, under optimal growth setting. Our results pointed out at significant differences of the exopolymers produced in the two conditions in terms of monosaccharidic composition and molecular weight distribution, and proved the capability of *S.* cf. *delicatissima* AMPL0116 to form stable bioaggregates on sandy soils.

Keywords Cyanobacteria · Biocrusts · Cyanobacterization · Microcosm inoculation · EPSs · Extracellular polymeric matrix

Introduction

The practice of cyanobacterization (use of cyanobacteria as soil fertilizers and conditioners) has been studied along the last 60 years, especially in order to increase crop yields from agricultural soils (Hamdi 1982; Priya et al. 2015; D'Acqui 2016). A successful inoculation procedure leads to the formation of organo-sedimentary layers at the topsoil (biocrusts), in which the action of cyanobacterial filaments and cyanobacterial-secreted exopolysaccharides (EPSs)

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determines a conglomeration of soil particles (Rossi and De Philippis 2015). Cyanobacterization can improve both soil structure (Maqubela et al. 2012) and soil fertility due to the release, by cyanobacteria, of bioactive substances, N, vitamins, polypeptides, and amino acids (Rodríguez et al. 2006). These positive effects are not only limited to the biocrust thickness but also extends to the subcrust (Guo et al. 2008).

Many studies concur in indicating cyanobacteria as valid eco-friendly biotechnological tools for improving the quality and harvest yields in arable lands and for the treatment of degraded and desertified environments (Wu et al. 2014; Lan et al. 2014; D'Acqui 2016). Their proven capability to withstand adverse climatic conditions make them feasible candidates even for the treatment of environments characterized by extreme drought, strong solar irradiation, and nutrient scarcity (Lan et al. 2014). Notwithstanding their limited differences in form, their stress tolerance and the response to environmental characteristics can vary when soil-inoculated. Indeed, Grant et al. (1985) observed that the success of cyanobacterization depends upon several variables, including soil properties and climatic and biotic factors. In this light, the selection of proficient inoculants according to the environmental characteristics and constraints is a key step for elaborating effective approaches. Notwithstanding the rather large number of cyanobacterial strains that have been tested as inoculants in laboratory and open field trials, there are still knowledge gaps regarding the physiological characteristics that make them good or bad candidates (Rossi et al. 2017).

Several studies indicated the excretion of EPSs as a key physiological process affecting the outcome of the inoculation process (Hu et al. 2003; Malam-Issa et al. 2007; Maqubela et al. 2010). One reason likely lies in that these mucilaginous exudates increase the inoculant resistance to adverse conditions (Pereira et al. 2009) and the extracellular polymeric matrix (EPM) in which they spatially organize in biocrusts constitutes an optimal microenvironment in terms of nutrient concentration and moisture balance (Or et al. 2007).

Additionally, and most importantly, EPS excretion improves soil stability (Hu et al. 2003; Malam-Issa et al. 2007; Maqubela et al. 2010) and moisture content at the topsoil (Colica et al. 2014), determining ameliorated conditions allowing a higher biological activity.

The importance of EPS productivity and characteristics as a screening criteria for inoculant selection is suggested by some studies (Brüll et al. 2000; Hu et al. 2003). However, these two parameters can be profoundly influenced by environmental conditions and abiotic stresses (Huang et al. 1998; Mager and Thomas 2010; Rossi and De Philippis 2015). It was suggested that cyanobacteria produce more chemically simple EPSs when growing under natural challenging conditions than when they are cultured in a laboratory with full availability of nutrients and optimal growth settings (Mager and Thomas 2011).

In order to evaluate the effect(s) of abiotic and nutritional stresses on EPS excretion in the cyanobacterization process, we compared the characteristics of EPSs produced by a desert-dweller strain, *Schizothrix* cf. *delicatissima* AMPL0116, when grown in liquid suspension and when dispersed on sandy soil in a microcosm experiment under water and nutrient stress, to simulate inoculation in an arid setting. The objectives of the study are to evaluate the differences in two key characteristics of EPSs: monosaccharidic composition and molecular weight (MW) distribution of EPSs produced in these two conditions, and to study, for the first time, the formation of EPM during the initial stages of biocrust development.

Material and methods

Isolation and identification of the cyanobacterium used as inoculant

The genus *Schizothrix* belongs to the order Oscillatoriales (Komárek et al. 2006) and has been commonly found in biocrust communities in arid and semiarid environments (Alwathnani and Johansen 2011; Dojani et al. 2014). Additionally, it was observed as a colonizer, in a network with cyanobacteria belonging to the genus *Microcoleus*, on unstable, coarse-grained substrates (Danin et al. 1998).

S. cf. *delicatissima* AMPL0116 was isolated from biocrusts collected at the edge of Hobq Desert, Inner Mongolia, China (40° 21′ 58″ N–109° 50′ 42″ E). The environment is characterized by moving sand dunes (average height 5 m) for 61% of the landscape. The climate is semiarid, temperate, and continental monsoon, with an average temperature of 6.1 °C and annual precipitation and evapotranspiration of 293 and 2448 mm, respectively (Rao et al. 2009).

Biocrusts were collected using a circle knife with a diameter of 7.5 cm. Crusts were transported to the lab and oven dried at 35 °C for 24 h. Crust fragments were immersed in sterile liquid BG11 medium (Rippka et al. 1979) under illumination of 20 μmol photons m^{-2} s^{-1} and at 25 °C, until green coloration was visible. Afterwards, liquid culture was repeatedly stricken on BG11-containing agar plates, maintained in the conditions described above. Single colonies were picked up with a sterile spatula from the agar and suspended again in liquid BG11 medium, in Pyrex flasks. The latter were incubated in an orbital incubator (Innova 44B, New Brunswick, USA) under continuous illumination (light intensity = 10–14 μmol photons m^{-2} s^{-1}). In order to purify the culture, it was transferred in BG11 medium supplemented with cycloheximide (purchased from Sigma Chemical Co.) at a concentration of 100 μg mL^{-1}, to eliminate eukaryotic contamination. The culture was then incubated in the dark for 22 h with three other antibiotics (ampicillin, rifampicin, and ciprofloxacin), which selectively killed growing heterotrophic bacteria

(Ferris and Hirsch 1991). The presence of only one cyanobacterial species was assessed by optical microscopic observations, while the possible presence of heterotrophic contaminants was excluded by plating the culture on agarized Luria-Bertani medium and then incubating it at 30 °C to observe possible colony formation. The identification of the strain was carried out by traditional (light microscopy). Samples of the culture were observed using several different light microscopy techniques (Axioskop and Stemi 200-C, Carl Zeiss, Jena, Germany), and genus and species were assigned by appropriate taxonomic keys (Komárek and Anagnostidis 1999, Komárek and Anagnostidis 2005).

The isolate was also identified by sequencing the 16S ribosomal DNA (rDNA) gene. Genomic DNA extraction was carried out using the protocol reported by Sinha et al. (2001). The amount of extracted DNA was quantified using NanoDrop (ND-1000, NanoDrop Inc., Wilmington, DE, USA). Bacterial 16S rDNA gene fragments were PCR-amplified using the cyanobacterial specific primers, forward primer CYA359F (5′-GGGG AATYTTCCGCAATGGG-3′), and an equimolar mixture of CYA781Ra (5′-GACTACT GGGGTATCTAATCC CATT-3′) and CYA781Rb (5′-GACTACA GGGGTATC TAATCCCTTT-3′) (Nübel et al. 1997).

PCR mixtures were set up in volumes of 50 μL and contained 10 μL Red Load Taq Master 5X (Larova, GmbH, Germany), 2.5 μL of each primer (CYA359F and CYA781R) (Integrated DNA Technologies, USA), 33 μL PCR grade H_2O (Larova, GmbH, Germany), and 2 μL of template DNA. The PCR cycling conditions were as follows: initial denaturation at 94 °C for 5 min, 30 cycles of denaturing at 94 °C for 30 s, annealing at 51 °C for 30 s, extension at 72 °C for 3 min, and a final extension for 2 min at 72 °C. Agarose gel electrophoresis (with addition of 10 μg mL^{-1} ethidium bromide) was used to check the size of the amplicons. The purification was made using the MinElute PCR purification Kit (Qiagen, Germany). Sequencing of the 16S rRNA was performed using the BigDye Terminator cycle sequencing chemistry from Applied Biosystems (ABI), ABI PRISM 3730xl DNA Analyzer and ABI's data collection and Sequence analysis softwares.

The cyanobacterium, identified as *S.* cf. *delicatissima* AMPL0116, was deposited at the Freshwater Algae Culture Collection (FACHB-collection) at the Institute of Hydrobiology of the Chinese Academy of Sciences, Wuhan, China, with deposit number FACHB2129.

Separation and characterization of EPSs obtained in liquid cultures

In order to produce the biomass for the inoculation in microcosms, the cyanobacterium was grown for 90 days in liquid cultures under the following conditions: a temperature of 30 °C, light intensity of 15 μmol photons m^{-2} s^{-1}, and a constant agitation of 100 rpm in an INNOVA 43R orbital incubator (INNOVA, New Brunswick). The released polysaccharides (RPSs) and the sheath were recovered from the liquid culture as follows: RPSs were extracted by centrifuging the culture at 4000×*g* for 30 min and concentrating the supernatant by evaporation. Afterwards, the supernatant was mixed with two volumes of cold (4 °C) isopropyl alcohol. After 8 h at 4 °C to allow the precipitation of RPSs, samples were centrifuged at 4000×*g* for 15 min and the pelletized RPSs resuspended in distilled water and lyophilized. Pelletized cyanobacterial cells after RPS recovery were washed with 1.5% NaCl solution and then extracted with 4 mL of distilled water at 80 °C for 1 h. After centrifuging at 4000×*g* for 30 min, the sheath-containing pellet was resuspended in distilled water and lyophilized. The effectiveness of the method in achieving a significant sheath extraction was checked and validated by optical microscope observations of the treated biomass in order to evaluate cell integrity and the significant presence of sheath-deprived filaments (Fig. S1).

The monosaccharidic composition of RPSs and of the sheath was determined by ion-exchange chromatography (IEC) (see the dedicated paragraph below). Before the analysis, 1 to 5 mg of lyophilized EPSs was hydrolyzed with 1 mL 2 N trifluoroacetic acid (TFA) at 120 °C for 120 min in screw-cap vials and then cooled on ice. Hydrolysates were filtered using centrifugal filters (AMICON Ultra-4, Billerica, MA) and evaporated 3 times in an orbital evaporator always resuspending in HPLC-pure water to remove TFA.

The MW distribution was determined by size exclusion chromatography (SEC), as described below in the dedicated paragraph, on the lyophilized EPSs previously solubilized in HPLC-grade water.

Inoculation of the strain in microcosms

Cyanobacterial biomass was inoculated in microcosms that constituted of two different types of plastic Petri dishes, having dimensions of 92 mm (diameter) × 16 mm (depth) and 60 mm (diameter) × 15 mm (depth) filled with 60 and 30 g sand, respectively.

In order to use an oligotrophic substrate typical of arid environments, sand was collected near Tlalim, Negev Desert, Israel (30° 58′ 05″ N–34° 38′ 10″ E). The environment where the sand was collected is hyperarid and characterized by sandy dunes with the presence of sparse subshrub vegetation. The sand, which has an alkaline pH (= 8.1), was collected far from any type of plants, on sandy dunes. Mobile sand is usually devoid of any coatings due to frequent abrasion by wind and has generally very limited C and N contents

(Felix-Henningsen et al. 2008; Roskin et al. 2011; Zaady et al. 2016). The sand was preventively sieved in order to work with a grain size ranging between 200 and 500 μm (medium sand, according to US Soil Taxonomy). It is well documented that biocrusts grow better on substrates with small granulometry (Rozenstein et al. 2014; Zaady et al. 2016), due to the higher field capacity/water availability.

Before inoculation, the cyanobacterial biomass produced in liquid cultures was separated from the culture medium by centrifugation at 4000×*g*, and the pelleted filaments were fragmented in sterile plastic tubes using a sterilized spatula. Eventually, the biomass was resuspended in distilled water in a volume calculated to provide sufficient inoculum for all the microcosms. Biomass was dispersed spirally on the microcosms, drop by drop, using a sterile 10-mL pipette, in volumes corresponding to 30 mg cell dry weight (CDW) (roughly corresponding to 2 g L^{-1} chlorophyll *a*) for the larger dishes and 15 mg CDW for the smaller ones. The spiral dispersion approach was conceived after several test trials with the aim to distribute the cells as uniformly as possible on the available surface of the microcosms. The quantity of the inoculum to apply was determined after preliminary test trials, applying increasing concentrations of biomass (from 10 to 30 mg CDW). While the lower concentrations produced inconsistent cyanobacterial crusts, concentrations in the higher range overflowed the microcosms, making it difficult to determine the actual crust development.

Inoculated microcosms were maintained inside a Plexiglas incubator with controlled temperature (30 °C) and light (45 μmol photons m^{-2} s^{-1}) for 90 days. Light was measured using a quantum meter (Spectrum Technologies, Plainfield, IL, USA). Relative humidity (RH) and temperature were monitored with a portable digital hygrothermometer (VWR, USA) having an accuracy of 5%, placed inside the incubation chamber. During the incubation time, each microcosm was provided with a total of 0.4 mm distilled water every 2 days, an amount roughly corresponding to the nonrainfall water abundance (dew) registered in the area where the soil was collected (Heusinkveld et al. 2006). Dew is present 200 nights per year, in amounts of 0.2–0.4 mL per square meters (mm) per day. In order to calculate the quantity of water to provide to each microcosm, we calculated the amount equivalent to a 3-month period and normalized it for the surface of the microcosms. Since a 0% RH was maintained in the growth chamber, the provided water amount represented the lone source for the microcosms. Noninoculated microcosms ($N = 3$) served as controls and were incubated under the same conditions and water provision as the samples.

Every 15 days, three large microcosms ($N = 3$) and three small microcosms ($N = 3$) were randomly collected to perform analyses regarding EPSs and growth parameters (chlorophyll *a*, total carbohydrates), respectively. All the analyses on biocrusts were performed after collecting the crusts with a clean spatula to a depth corresponding to their thickness (see Fig. 2c), homogenized in a mortar, and weighed.

Measurement of total carbohydrate and chlorophyll content of the biocrusts

Total carbohydrates were determined by suspending 30–50 mg homogenized crust in 1 mL distilled water and then applying the phenol-sulfuric acid method (Dubois et al. 1956) as follows: 1 mL of sample suspension was mixed with 1 mL 5% phenol, followed by 5 mL H_2SO_4 in screw-cap glass vials for 10 min. Afterwards, vials were dipped in cool water for 15 min and then the reaction mix was analyzed by determining the absorbance at 488 nm with a UV-VIS spectrophotometer (Varian, Cary 50). Calibration was performed using D-glucose at different concentrations as a reference standard.

Chlorophyll *a* in biocrusts was determined as reported by Castle et al. (2011): 1 g of homogenized crust was treated with 5 mL ethanol at 80 °C for 5 min and then incubated at 4 °C for 8 h. Subsequently, samples were centrifuged at 3500×*g* for 15 min and the pigment-containing supernatant was recovered. The extraction method was applied twice to each sample in order to achieve a full pigment recovery. Chlorophyll *a* content was determined by measuring the absorbance (*A*) at 665 nm. Each value was corrected ($A_{(665c)}$) by subtracting the absorbance at 750 nm to account for residual scattering of the solution. Chlorophyll *a* concentration was calculated by applying the formula reported by Ritchie (2006), taking into account the amount of crust (g crust) that was treated:

$$\mathrm{Chl}a\left(\mathrm{mg\ L^{-1}}\right) = \frac{[11.9035 \times A(665c)] \times V}{(\mathrm{g}\,crust) \times L}$$

where *V* is the volume of the extract and *L* is the optical path length.

Measurement of soil water repellency

For the determination of soil water repellency (SWR), we used two different methods. For the first, we employed a miniaturized tension infiltrometer as described by Lichner et al. (2013) to determine the repellency index (RI). Here, we measured the sorptivity of water and ethanol for each sample and then calculated the RI using the following formula:

$$\mathrm{RI} = 1.95\sqrt{\frac{S_\mathrm{E}}{S_\mathrm{W}}}$$

where S_E is the sorptivity of ethanol and S_W is the sorptivity of water, and the factor of 1.95 accounts for differences in viscosity and surface tension between the two liquids (Tillman et al. 1989). A soil with a RI = 1 (i.e., where $S_E = S_W$) is considered nonrepellent, whereas a subcritical repellency is characterized by RI values greater than 1, but finite

(according to Tillman et al. 1989, S_W in a critically repellent soil will be 0, so the RI will be infinite). Further, we determined the contact angle (CA) of the soil using the Wilhelmy plate method (Bachmann et al. 2003) on disturbed samples using a contact angle tensiometer (DCAT 11, DataPhysics, Filderstadt, Germany), measuring the advancing contact angle. Both measurements were performed at least in triplicates.

Extraction, quantification, and characterization of loosely bound EPSs and tightly bound EPSs

The EPSs were extracted from the biocrusts grown in microcosms as two operationally defined fractions: one less condensed and more water soluble (loosely bound EPSs, LB-EPSs) and one having a higher level of gelification and being more tightly attached to cells and sediments (tightly bound EPSs, TB-EPSs). The partitioned extraction is meant to evidence eventual differences between the two fractions which, according to a previous study (Chen et al. 2014), likely play different roles within the crust system. The two fractions were extracted slightly modifying the methods reported by De Brower and Stal (2001), Rossi et al. (2012), and Chen et al. (2014).

Modifications from the published methods consisted of including repetitions of the extraction procedures to achieve consistent fraction recovery. In order to recover LB-EPSs, homogenized crusts were extracted with distilled water at room temperature for 15 min. Next, samples were centrifuged at 3500×*g* for 30 min to recover the LB-EPS-containing supernatants. For each experimental replicate, the water extraction procedure was repeated five times, and the supernatants from each replicate (*N*) were collected together. In order to recover TB-EPSs, the resulting pellet was treated with 5 mL 0.1 M Na_2EDTA for 16 h at room temperature before centrifuging at 3500×*g* for 15 min. The extraction was repeated three times in total on each replicate (*N*), the latter two extractions protracted for 120 min each. Eventually, the three supernatants from the three sequential extractions were collected together.

LB-EPSs and TB-EPSs in the extracts were quantified by applying phenol-sulfuric acid assay, previously described, on 1 ml aliquots of the extracts. No chlorophyll *a* was found in the extracts, suggesting that the extraction procedures did not damage cells causing the leakage of intracellular carbohydrates.

The monosaccharidic composition of LB-EPSs and TB-EPSs was determined by IEC as described in the following paragraph. Before IEC analysis, the extracts were hydrolyzed adding 1 part of extract to 1 part of 4 N TFA in screw-cap vials, for 120 min, at 120 °C. In the case of TB-EPSs, prior to the hydrolysis, extracts were put in 12–14 k MW cutoff nitrocellulose dialysis tubes (Medicell International Ltd., London) and dialyzed for 24 h against distilled water to remove Na_2EDTA that could interfere with the chromatographic analysis.

Apparent MW distribution of the two fractions was determined centrifuging EPS extracts at 13,000×*g* in Eppendorf tubes to remove the coarse particulate and then analyzed by SEC as described in the following paragraph.

Chemical and macromolecular characterization of the EPSs

After the hydrolytic procedures reported above, the EPSs obtained in liquid cultures or in microcosms were analyzed for their monosaccharidic composition by a Dionex ICS-500 chromatographer (Dionex, Sunnyvale, CA), equipped with an ion-exchange column (CarboPac PA1) and an ED 50 electrochemical detector with a gold-working electrode. Chromatographic conditions were those reported by Chen et al. (2014). The eluents used were Milli-Q-grade water (A), 0.185 M sodium hydroxide (B), and 0.488 M sodium acetate (C). In the first stage of the analysis (from injection time to 7 min), the eluent was constituted by 84% A, 15% B, and 1% C; in the second stage (from 7 to 15 min), the eluent was constituted by 0% A, 50% B, and 50% C; in the final stage (from 15 to 30 min), the eluent was that of the first stage. The working flow was 1.00 mL min^{-1} with running times of 30 min. Response factors of each sugar were determined by injecting known concentrations of pure monosaccharide standards (purchased from Sigma-Aldrich).

The MW distribution was determined by injecting the samples in a Varian Pro-Star liquid chromatographer (Varian Inc., USA) equipped with two PolySep-GFC-P 6000 and 4000 columns (Phenomenex, USA) connected in series and a refractive index detector. The eluent was HPLC-grade water at a working flow of 0.4 mL min^{-1}. Dextran at known MWs (2, 1.1, 0.41, 0.15, 0.05 M), purchased from Sigma-Aldrich, was used as standards.

Statistical analysis

Data sets were compared by applying Student's *t* test or one-way analysis of variance (ANOVA) at the 95% significance level. Every measurement was performed in instrumental triplicates ($N = 3$) for which a mean value was calculated. Mean values and SDs were calculated at least on three different independent replicates (statistical replicates, $N = 3$). Where needed, regression analysis to correlate parameters was done, reporting r^2 and *P* values in each case. For all statistical analysis apart from RI and CA measurements, GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA, USA) was used. For the statistical analysis of IR and CA, Student's *t* test was used with Microsoft Excel, version 2013.

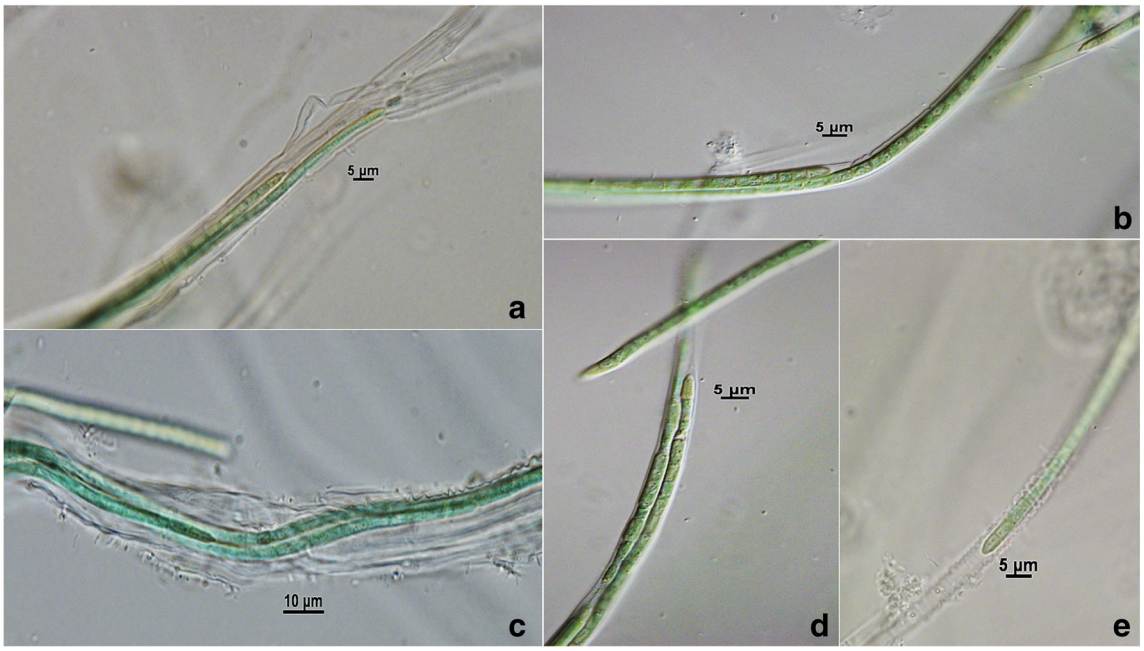

Fig. 1 **a**–**e** Optical microscope images of *S.* cf. *delicatissima* AMPL0116 evidencing the sheath

Results

Identification of the strain

The morphology of the strain allowed to identify it as *Schizothrix* cf. *delicatissima* (Fig. 1). It is characterized by solitary, sometimes pseudobranched trichomes (100–600 μm long, 5–6.5 μm thin) (Fig. 1a, b) surrounded by a thick smooth sheath (Fig. 1c) containing 1–2 trichomes (Fig. 1d). The sheath is wavy and sometimes lamelled (Fig. 1e). Cells are (4)6–8(10)× longer than wide.

The DNA sequence of the 16S rRNA gene obtained from this study was deposited in GenBank with accession number KY120205. 16S rDNA analysis showed 98% identity to the 16S sequence of *Schizothrix* sp. UAM 402 (KF544967) and *Schizothrix* sp. UAM 404 (KF544969) from the NCBI 16S

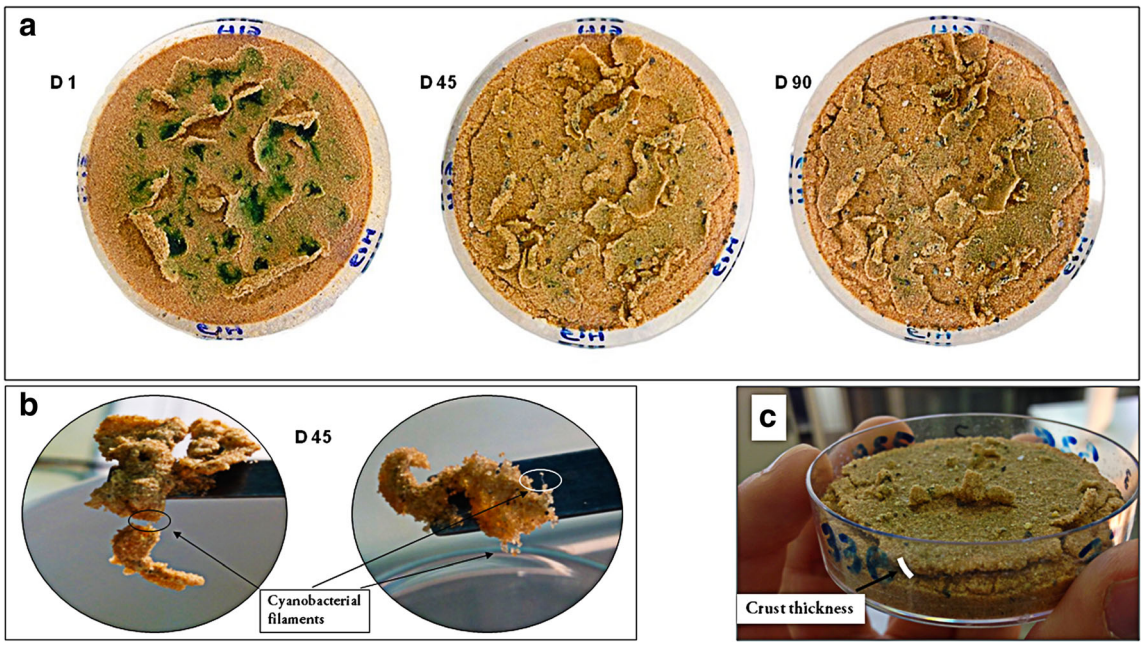

Fig. 2 **a** Development of cyanobacterial crusts from the day after inoculation (day 1, D1), halfway in the incubation time (day 45, D45), and at the end of incubation time (day 90, D90). **b** Detached 45-day-old crust shred, disclosing the presence of cyanobacterial bundles. **c** Semi-side view picture of a 90-day-crust showing a thickness of approximately 2 mm

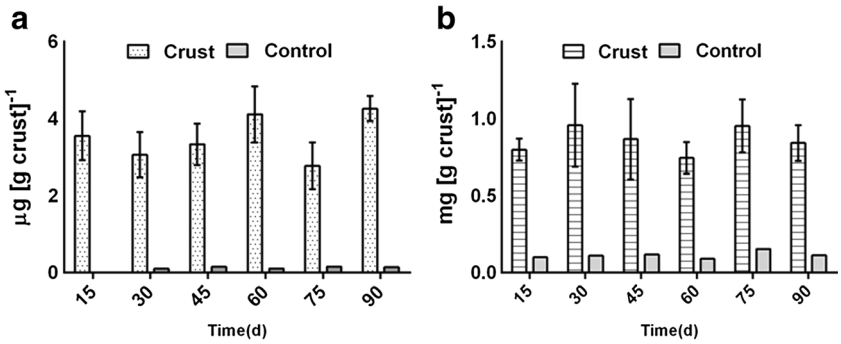

Fig. 3 **a** Chlorophyll *a* and **b** total carbohydrate content in cyanobacterial crusts induced by inoculation of *S.* cf. *delicatissima* AMPL0116 (values represent the mean of $N = 3$, error bars represent SD). No significant difference was found between values along the incubation period, in the case of chlorophyll *a* and in the case of total carbohydrate content

Ribosomal RNA Sequences Database using BLAST (Altschul et al. 1990).

Development of biocrusts

The inoculation of *S. delicatissima* AMPL0116 produced visually evident biocrusts (Fig. 2a), with obvious modification from the day after the inoculation to the end of the incubation period. At day 1, the spiral inoculation path on the surface of the microcosm was still visible. The first clear signs of sand aggregation started to appear, with cyanobacterial biomass forming flakes and determining upturned/curled features of the microbial-sediment structures. At day 45, crusts presented diffused cracks on the surface, and crusting borders appeared extended to reach the microcosm limits. All these characteristics accentuated in 90-day-old crusts. A close examination of pieces of crusts unveiled the massive presence of filament bundles collating aggregates (Fig. 2b).

A side view observation of the crusts allowed observing horizontal structuring, with demarked thickness limits of roughly 2 mm (Fig. 2c).

Chlorophyll *a* and total carbohydrate content of biocrusts

Chlorophyll *a* content in the crusts did not show significant variations during incubation time (Fig. 3a). The analysis of variance ruled out the significance of the observed fluctuations, averagely between 3.5 and 4.3 µg (g soil)$^{-1}$ ($P = 0.052$).

Similarly, total carbohydrate content did not vary significantly ($P = 0.683$), ranging averagely from 0.74 to 0.96 mg per gram of crust (Fig. 3b). As expected, the values of the two parameters were significantly higher in the biocrusts, in comparison with the controls.

Soil water repellency

The CAs did not increase during the entire experiment and no significant differences were observed between the crust and control at any time (Fig. 4). A soil is considered water repellent if the CA exceeds 90°, but the fact that CAs in our experiment were well below 20° at any time shows that no SWR was induced due to cyanobacterial growth.

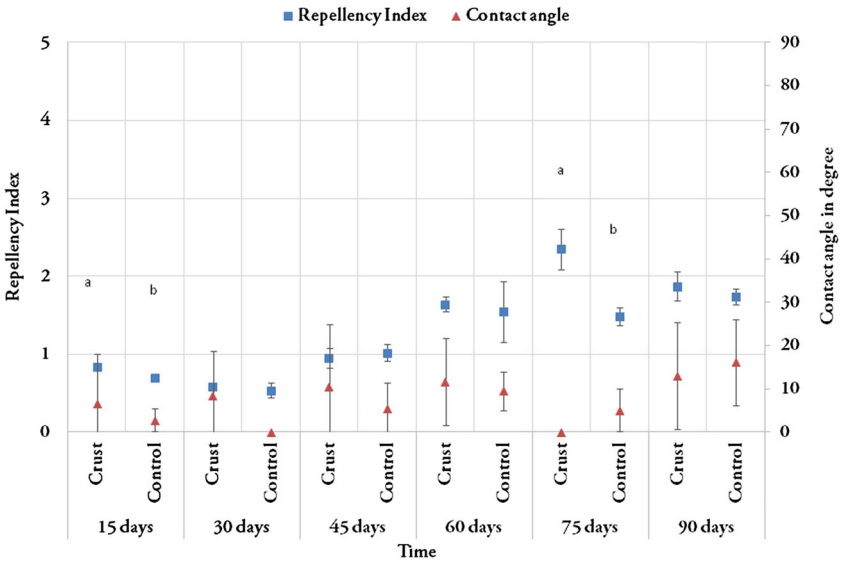

Fig. 4 Soil water repellency of the crusts. Both repellency indices (squares) and contact angles (triangles) show no strong effect of the inoculation on Error hydrophobicity over time. bars symbolize standard hydrophobicity over time. deviations of at least $N = 3$. Different letters indicate hydrophobicity over time. significant differences (only the case of RI)

Table 1 Monosaccharidic composition (expressed as moles of the single monosaccharide divided by the total amount of moles of monosaccharides in the EPS × 100)

Monosaccharidic composition (moles %)		
	RPSs	Sheath
Fuc	tr	0.64 (0.24)
Rha	tr	0.84 (0.61)
GalN	0.96 (0.53)	0.20 (0.05)
Ara	4.47 (0.54)	11.69 (1.01)*
GlcN	8.15 (1.94)	nd
Gal	11.30 (0.84)	11.22 (2.38)
Glc	53.54 (0.51)*	40.86 (0.57)
Xyl	8.36 (1.44)	20.05 (1.03)*
Fru	0.32 (0.19)	0.78 (0.46)
Rib	5.80 (1.21)*	1.57 (1.21)
GalA	nd	1.26 (0.28)
GlcA	5.44 (1.30)	10.87 (1.39)*

Contents labeled as *tr* were found not in all the biological replicates and always in contents smaller than 1%. Values are means of three determinations ($N = 3$) and SDs are reported in brackets. Values marked thus * are significantly higher than their counterpart in the other fraction

Fuc fucose, *Rha* rhamnose, *GalN* galactosamine, *Ara* arabinose, *GlcN* glucosamine, *Gal* galactose, *Glc* glucose, *Man* mannose, *Xyl* xylose, *Fru* fructose, *Rib* ribose, *GalA* galacturonic acid, *GlcA* glucuronic acid, *nd* not detected, *tr* detected only in traces

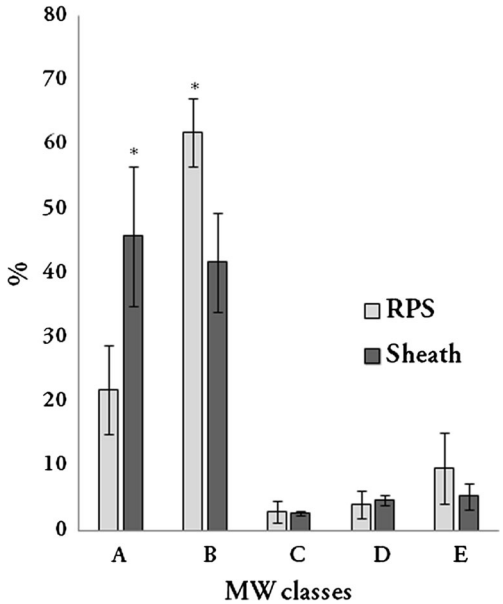

Fig. 5 Molecular weight distribution of RPS and sheath of *S.* cf. *delicatissima* AMPL0116 within 5 MW classes (A–E). A, higher than 2 MDa; B, between 2 and 1.1 MDa; C, between 1.1 MDa and 410 kDa; D, between 410 and 150 kDa; E, between 150 and 50 kDa. Values are means of three determinations. Values marked with * indicate a significant difference between the RPS and the sheath

The same is true for the RI, with the slight difference that a small increase was observed over time and that mean comparisons between control and crust yielded significant differences in two cases, namely at 15 and 75 days (Fig. 4). However, in most cases, RI was (close to 1) below 1.95 indicating nonrepellent soil crusts, and only for the crust at 75 days of incubation time, RI is slightly higher than 1 corresponding to subcritical water repellency. The results of RI are, therefore, generally consistent with the very low values for the CA.

Characterization of released and sheath polysaccharides in liquid culture

EPSs excreted by *S. delicatissima* AMPL0116 in liquid cultures were composed of 12 different types of monosaccharides: dehoxy-sugars, fucose and rhamnose; pentoses, arabinose, xylose, and ribose; aldo-hexoses, glucose and galactose; cheto-hexose, fructose; amino-sugars, galactosamine and glucosamine; and acidic sugars, galacturonic and glucuronic acids. Overall, glucose, galactose, xylose, arabinose, glucosamine, ribose, and glucuronic acid were the sugars detected at higher relative percentages (Table 1). Globally, galactose, glucose, and xylose represented 73.20 and 72.13% of the relative abundance in the RPSs and in the sheath, respectively. However, the two fractions showed some pronounced differences. While the former does not contain galacturonic acid, the latter does not contain glucosamine. In addition, some constituents showed different internal molar percentages (Table 1).

SEC analysis showed that both the RPSs and the sheath were mostly composed of high MW fractions (Fig. 5). The total percentages of the two fractions with a MW between 1 and 2 MDa and with a MW higher than 2 MDa accounted for more than 80% and for nearly 90% in RPSs and in sheath, respectively. However, the RPSs and the sheath differed significantly for what concerns the ratio between the two molecular fractions having the highest MW. Unpaired *t* test comparison ruled that while RPSs were constituted by a higher number of molecules with MW in the range between 2 and 1.1 MDa ($P = 0.010$), the sheath was constituted by a higher number of molecules with a MW higher than 2 MDa ($P = 0.015$).

Quantification and characterization of EPS in biocrusts

The amount of EPSs extracted from biocrusts (i.e., LB-EPSs plus TB-EPSs) increased during the whole incubation time, from 93.65 ± 8.37 to 205 ± 19.97 μg, per gram of crust. The increment of EPSs is due to the increase of the LB fraction. This fraction increased significantly, from roughly 43.6 (day 15) to around 108 μg per gram of crust (day 90) (Fig. 6a) with a positive correlation with the age of biocrusts ($r^2 = 0.69$,

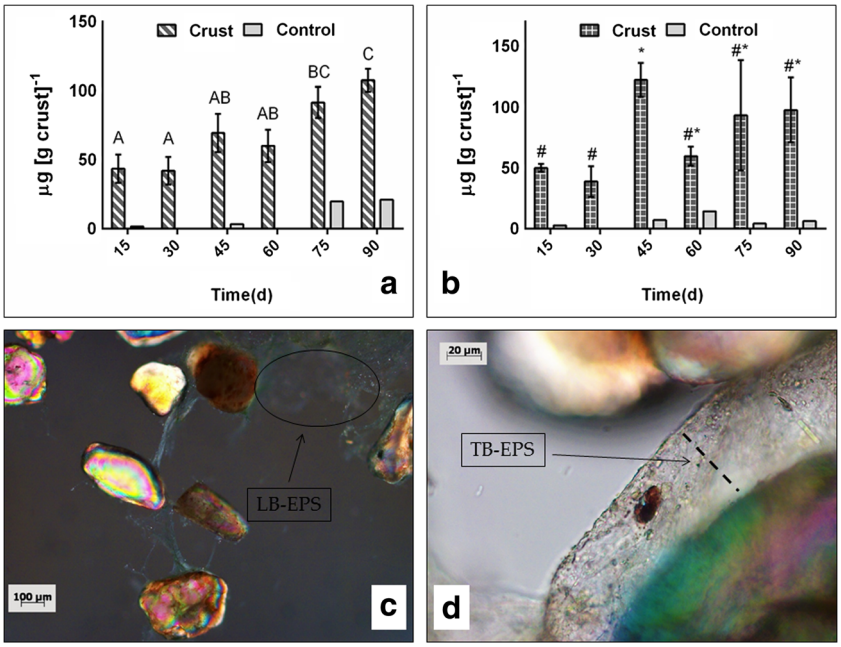

Fig. 6 LB-EPS (**a**) and TB-EPS (**b**) contents of cyanobacterial crusts during their development. Values are the means of three replicates. Values marked with different symbols are significantly different ($P < 0.05$). **c** Optical microscope image showing sand particles embedded in EPS and cyanobacterial filaments; hydrophobicity over time. LB-EPSs are pointed out. **d** Close-up of a sand grain covered by TB-EPSs. The pictures were taken on cyanobacterial crusts of 90 days

$P < 0.01$). During the whole incubation time, the analysis of variance pointed out that the content of TB-EPSs did not vary significantly ($P = 0.001$), although t test analysis between values at 15 and 90 days showed a significant increase ($P = 0.019$), from roughly 50 to 95 µg per gram of crust (Fig. 6b).

Optical microscope observations conducted on a 90-day biocrust showed distinctively the presence of LB-EPSs (Fig. 6c) and TB-EPSs (Fig. 6d) constituting the EPM. After 15 days of incubation, EPSs extracted from biocrusts were prominently composed of glucose, galactose, and xylose (Table 2), the three accounting overall for roughly 85% of LB-EPSs and 87% of TB-EPSs. Compared to liquid suspension, arabinose, ribose, and uronic acids were detected in lower relative amounts, with uronic acids detected only in traces. On the contrary, rhamnose resulted in higher relative abundances throughout the incubation time.

LB-EPSs and TB-EPSs showed significant compositional differences. Besides the three dominant neutral sugars, the former contained arabinose and glucosamine, with other constituents detected only in traces. TB-EPSs showed conversely substantial amounts of fucose, rhamnose, and galactosamine and higher relative abundance of xylose. At the end of the incubation time (day 90), TB-EPSs showed essentially galactose, glucose, and xylose (representing overall roughly 90% of the relative abundance) and rhamnose (6.51%), while the other sugars were detected only in traces. In addition to the three dominant neutral sugars, LB-EPSs contained arabinose (4.08%) and glucosamine (8.36%).

SEC analysis showed that EPSs extracted from biocrusts had varying MWs (Fig. 7) with LB-EPSs and TB-EPSs having markedly different MW distribution profiles. LB-EPSs resulted composed of molecules with an apparent MW in the range 2–1.1 MDa, roughly from 54 to 90%. TB-EPSs resulted averagely from 38 to 45% molecules with an apparent MW between 150 and 50 kDa, for roughly 26 to 30% molecules between 410 and 150 kDa, for 10% between 1.1 MDa and 410 kDa, and for roughly 16 to 20% between 2 and 1.1 MDa. Another difference between the fractions was represented by molecules with an apparent MW higher than 2 MDa. While in LB-EPSs they represented from 7 to 15%, in TB-EPSs, they were detected, for the most, in traces.

Discussion

This study contributes to implement our understanding of the interactions existing between microorganisms and minerals at biogeochemical interfaces in soil, choosing a methodology that can be encompassed between the so-called artificial soil maturation experiments (Pronk et al. 2017). In particular, we worked toward examining biotic and abiotic modifications of a sandy substrate after the introduction of a cyanobacterial inoculant in a designed laboratory study.

The inoculation of *S. delicatissima* AMPL0116, carried out following a dispersal procedure that was optimized for the purpose, induced stable cyanobacterial crusts. Inoculated microcosms were maintained throughout the experiment under continuous illumination, only providing a minimal amount of water. Notwithstanding these highly stressing conditions, the inoculated biomass organized in sheets of filaments and aggregated sand. During incubation time, the biosedimentary structures formed upward curls and an increasing number of cracks. In natural conditions, the

Table 2 Monosaccharidic composition (expressed as moles of the single monosaccharide divided by the total amount of moles of monosaccharides in the EPS × 100) of LB-EPSs and TB-EPSs extracted from biocrusts of different ages

Monosaccharidic composition (moles %)												
Time (days)	15		30		45		60		75		90	
	LB-EPSs	TB-EPSs	LB-EPSs	TB-EPSs	LB-EPSs	TB-EPSs	LB-EPSs	TB-EPSs	LB-EPSs	TB-EPSs	LB-EPSs	TB-EPSs
Fuc	tr	1.74 (1.44)	tr	tr	1.18 (0.26)	1.23 (0.17)	tr	tr	tr	tr	tr	tr
Rha	tr	4.76 (2.43)	tr	4.66 (3.00)	1.13 (0.27)	7.09 (2.66)	3.52 (2.40)	4.08 (3.11)	3.86 (1.54)	6.64 (3.03)	tr	6.51 (5.64)
GalN	tr	1.60 (1.08)	tr	2.95 (0.86)	4.22 (4.60)	1.39 (1.53)	1.41 (1.30)	tr	1.45 (0.67)	1.65 (0.37)	tr	tr
Ara	3.90 (0.58)	tr	2.76 (1.88)	1.08 (0.21)	3.06 (0.41)	1.51 (1.35)	4.97 (2.83)	tr	4.64 (1.04)	tr	4.08 (1.96)	tr
GlcN	6.72 (3.12)	1.33 (0.93)	9.31 (5.04)	tr	11.67 (3.25)	1.19 (0.91)	8.04 (2.80)	tr	14.25 (2.00)	tr	8.36 (3.48)	tr
Gal	25.87 (0.16)	19.09 (5.67)	22.25 (0.29)	20.50 (2.00)	17.49 (11.68)	21.75 (4.36)	14.81 (4.40)	22.92 (5.12)	22.60 (8.76)	24.43 (2.23)	24.92 (5.72)	22.85 (6.43)
Glc	57.64 (10.18)	59.75 (2.40)	55.80 (9.00)	61.56 (6.81)	50.38 (6.67)	56.15 (2.38)	53.73 (3.41)	58.42 (5.07)	44.15 (9.78)	55.87 (0.79)	51.56 (9.06)	57.89 (0.00)
Xyl	2.10 (0.98)	8.10 (1.75)	4.11 (2.42)	6.20 (0.11)	4.21 (0.85)	8.03 (2.50)	5.11 (1.43)	8.04 (0.24)	7.65 (1.20)	8.28 (0.47)	7.47 (3.26)	8.99 (2.64)
Fru	tr	1.18 (0.92)	2.99 (3.12)	tr	4.82 (3.09)	tr	1.76 (1.54)	tr	tr	tr	tr	tr
Rib	tr	1.34 (1.20)	tr	1.40 (0.62)	1.84 (1.81)	1.09 (1.37)	0.18 (0.31)	tr	tr	tr	tr	nd
GalA	tr	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
GlcA	nd	tr	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

Values represent the mean values from $N = 3$ and are expressed as mean (±SD). Contents labeled as *tr* (traces) were found not in all the biological replicates and always in contents lower than 1%, averagely *nd* not detected

morphology of the biocrusts strictly depends on the characteristics of the soil and on climate (Belnap et al. 2001). Morphological characteristics similar to those observed in our experiment were found in biocrusts in Mohave Desert (Williams et al. 2012). The formation of cracks was explained as due to shrinking and swelling of clay minerals (Williams et al. 2012) and to the viscoelastic properties of EPSs (Navarini et al. 1992), while similar curls were observed upon drying in cyanobacteria-dominated biocrusts (Skujins 1991). In natural dry hot environments, the formation of cracks provides open pathways to increase soil permeability and aeration under the sheets (Williams et al. 2012), leading to new colonizable niches with ameliorated moisture regimes (Danin et al. 1998). In our study, filament bundles became visible when biocrusts were fragmented, testifying the colonization capability of the strain, which was originally dispersed only in fragmented form.

At the same time, our data suggest a restrained growth, testified by the statistically nonsignificant increase in chlorophyll *a* and total carbohydrate content during the incubation time. We assume that the absence of growth found in our study was due to the very limited water supply and very low humidity. Nonetheless, in a similar microcosm experiment employing the Oscillatorean strain *Microcoleus vaginatus* in less stressing conditions, the authors also observed a nonsignificant increase of proteins, chlorophyll, and carbohydrates (Rozenstein et al. 2014). The same authors suggested the nonreliability of these parameters to measure the inceptive colonization of biocrusts, showing the greater consistency of techniques such as electronic microscopy or reflectance spectroscopy.

In this study, microscopical observations of the dismantled crust structure clearly showed the presence of a mucous-dispersed fraction (which we referred to as LB-EPSs) and a fraction enveloping, or strictly connected to, filaments and sand grains (which we referred to as TB-EPSs). Our data showed a constant increase of the former less condensed and more soluble fraction, in correlation with incubation time. Apparently, stressing conditions elicited EPS synthesis at the expense of cell growth. The stimulating effect of a reduced moisture level on extracellular carbohydrate synthesis has already been reported by Mazor et al. (1996). This is not surprising considering the role of EPSs in increasing water-stress tolerance and their contribution in crust-water relations (Hill et al. 1994; Colica et al. 2014).

Crust development did not further increase in water repellency. A soil with a CA between 0° and 90° was defined by Lamparter et al. (2006) as "subcritically water repellent." Concerning biocrusts in the Negev Desert, Keck et al. (2016) reported subcritical SWR with similar CAs. Since the values for RI and CA of our study were even smaller, we can conclude that crust inoculation caused SWR only to a very small, subcritical degree in our samples. Most probably, very

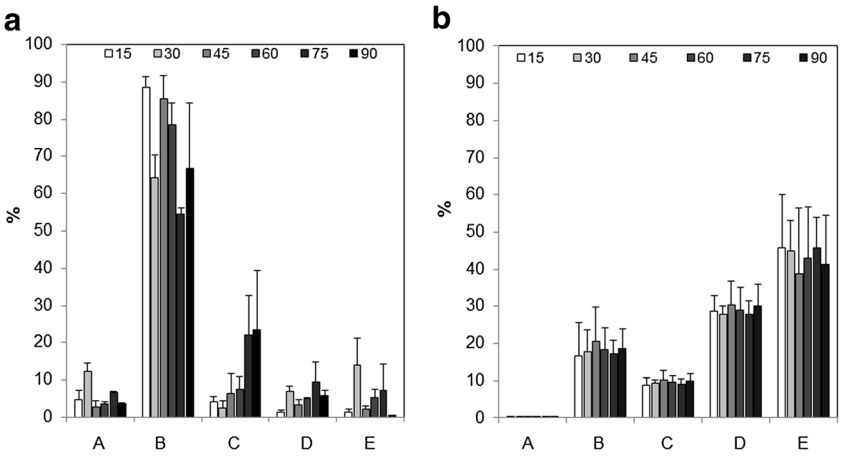

Fig. 7 **a** MW distribution of LB-EPSs and **b** TB-EPSs within the five weight classes (A–E) during cyanobacterial crust development (evaluated after 15, 30, 45, 60, 75, and 90 days). A, higher than 2 MDa; B, between 2 and 1.1 MDa; C, between 1.1 MDa and 410 kDa; D, between 410 and 150 kDa; E, between 150 and 50 kDa ranges. Values are means $N = 3$ and SDs are reported in the figure

young crusts, having only 3 months of age, are not yet capable of causing significant changes in a macroscopic feature like SWR.

When cultured in liquid suspension, *S.* cf. *delicatissima* AMPL0116 excreted compositionally rich EPSs, which are partly released in the medium (RPSs), and partly constitute the thick sheath surrounding the trichomes. RPSs and sheath resulted to be compositionally similar, with minimal differences. Both EPS fractions also share the characteristic of being mostly composed of macromolecules with a MW higher than 1 MDa, thus confirming that most cyanobacterial EPSs are characterized by high MW (Pereira et al. 2009).

EPSs extracted from the biocrusts showed a compositional pattern similar to that produced by the strain in liquid culture. Notably, galactose, glucose, and xylose resulted to be the most abundant components, indicating that they represent the polysaccharidic "core" of the EPSs produced by this strain. At the same time, some components were detected in different relative abundances.

Compared to EPSs produced in liquid culture, EPSs from biocrusts showed a higher content in rhamnose, detected mainly in TB-EPSs, while it showed lower abundances of uronic acids and arabinose. The higher content in rhamnose, which is a deoxysugar having a hydrophobic character that favors the attachment to solid surfaces (Pereira et al. 2009), may explain the tendency of the TB-EPSs to be tightly bound to sand particles. Interestingly, while sugars composing LB-EPSs were in similar internal molar ratio throughout the incubation period, TB-EPSs bore significant differences. While the "core" components and rhamnose had similar abundances from 15 to 90 days, other components were only detected in traces at the end of incubation time. Most probably, being in oligotrophic and abiotic stressing conditions, the strain produced compositionally simpler exopolymers compared to the liquid culture, where it experiences optimal abiotic conditions and excess of nutrients, as suggested by Brüll et al. (2000).

Studying the early development stages of biocrusts, Zhang (2005) observed that cyanobacterial sheaths deprived of the inner producing filament (that either migrated or died) provided the major contribution to sediment cohesion, appearing in condensed form attached to sand grains. In this view, TB-EPSs might derive from the sheath material produced by *S. delicatissima*. Stressing conditions seem to restrain the synthesis of this fraction while they do not seem to affect the synthesis of LB-EPSs. If this is the correct interpretation, this study confirms the previously hypothesized influence that nutrient status (notably the availability of N and P) and abiotic stress have on EPS excretion (Huang et al. 1998; Brüll et al. 2000). At the same time, these results point at a possible different excretion mechanism of LB-EPSs, and TB-EPSs, evidenced by the analysis performed during incubation time.

The same factors impinging on the differences in composition might be the cause of the differences in the MW distribution profiles of EPSs between liquid culture and microcosms. The more variegate distribution profile in microcosm, i.e., the conspicuous higher presence of polymers with apparent MWs lower than 1 MDa, is compatible with a downregulation of those enzymes involved in polymer assembly and elongation (Pereira et al. 2009). Again, this is true for TB-EPSs, while LB-EPSs do not seem to be affected by different growth conditions, maintaining a MW distribution profile similar to that observed in liquid culture.

This study provides, for the first time, an insight on the chemical and macromolecular characteristics of EPSs produced by cyanobacterial inoculants in the first stages of their colonization of sandy soil. The EPS synthesis appeared significantly altered under stressing conditions in soil compared to liquid culture, both in composition and MW distribution, with special regard to the more condensed EPS fraction (TB-EPSs). No alterations were observed for the more dispersed and soluble fraction (LB-EPSs). This suggests an active role of the latter fraction in fostering the formation of cyanobacterial crusts under stressing conditions, with its

synthesis representing the larger investment for the strain in the conditions in which this experiment was carried out.

Notwithstanding its restrained growth, the strain *S.* cf. *delicatissima* AMPL0116 proved to be a good candidate as inoculant and deserved further studies.

Conclusions

In conclusion, this study clearly showed that the process of colonization of sandy soil started immediately after the inoculation. *S. delicatissima* was capable of establishing a relatively thick crust in a very short period of time, releasing a rather large amount of EPSs in a few weeks. On the other hand, the period of time was probably too short for inducing also a change in the hydrological properties tested, possibly because the distribution of the trichomes and of the EPSs on the surface and on the bulk of the crust was not yet homogeneous enough.

The result of this study is that the chemical and macromolecular characteristics of the EPSs produced in liquid cultures under optimal growth conditions or in the sandy soil under stress conditions are significantly different. Moreover, the two EPS fractions, TB-EPSs and LB-EPSs, interestingly showed significant differences, particularly in their MW distribution. These previously unreported observations clearly point out the need of investigating on how the expression of the genes involved in EPS biosynthesis is regulated by the environmental conditions in cells growing in sand.

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