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RESEARCH ARTICLE

Isolation and characterization of soil cyanobacteria and microalgae and evaluation of their potential as plant biostimulants

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Abstract

Background and Aims Biocrusts are found on soil surface resulting from an association between soil particles and microorganisms. Photoautotrophic cyanobacteria and microalgae are pioneers on biocrusts formation, promoting soil stability, nutrients availability and water retention, leading to the development of other communities. This work aimed at isolating and characterizing cyanobacteria/microalgae from biocrusts (Central Portugal) and to assess their potential as plant biostimulants, as well as obtaining an insight into their mechanism(s) of action.

Methods Microorganisms were isolated through successive spread plating/serial dilutions and characterized using genetical analysis/morphological traits. An initial screening was performed using exudates from each microorganism and two plant species, *Arabidopsis thaliana* and *Lolium multiflorum*. Subsequently, the selected microorganisms were tested as a consortium in hydroponic systems. Biometric and biochemical parameters were evaluated for both plant species.

Results The consortium microorganisms belong to genera often found in soils/biocrusts: *Trichocoleus, Nodosilinea, Microcoleus* (filamentous cyanobacteria), *Nostoc* (diazotrophic heterocystous cyanobacteria), and *Klebsormidium* (filamentous microalga), and some of them have the capacity to produce phytohormones and/or siderophores. The consortium showed biostimulant potential in hydroponic cultures, promoting plant growth and enhancing physiological productivity related

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parameters. Stress related parameters revealed that the microorganisms did not lead to a stressful situation. However, a significant increase in proline was observed, endorsing a role of this molecule in this process.

Conclusion This study contributes to the knowledge on the biodiversity of cyanobacteria and microalgae from Portuguese soils and highlights their potential as biostimulants, constituting a step forward towards understanding the molecular mechanisms behind this effect.

Keywords Biocrusts · Biostimulants · Cyanobacteria · Microalgae · Plant growth

Introduction

Biological soil crusts (or biocrusts) are found in the upper millimeters of the soil and result from an intimate association between soil particles and different proportions of photoautotrophic (e.g. cyanobacteria, algae, lichens, bryophytes) and heterotrophic (e.g. bacteria, fungi, archaea) organisms (Weber et al. 2022). These biocrusts have crucial roles on soil ecosystems by regulating soil hydrology, promoting soil stabilization, conferring protection against erosion and increasing soil fertility (Ferrenberg et al. 2017). The biocrust succession starts with the colonization of the bare soil by the photoautotrophic microorganisms to form cyanobacterial/algae crusts. Subsequently, the soil stability and carbon and nitrogen levels increase allowing further successional stages, namely the appearance of lichen and moss crusts (Bao et al. 2019; Rossi et al. 2017; Yang et al. 2022; Zhang et al. 2018). From this point onwards, the conditions for the development of other communities (such as shrubs and trees) are established, allowing the implementation of a stable ecosystem (Chamizo et al. 2012; Ferrenberg et al. 2017).

Over the last years, and since cyanobacteria and microalgae are the pioneers for biocrusts' formation, several studies focusing on the inoculation of soils with these microorganisms (cyanobacterization/algalization) have been carried out (Çakirsoy et al. 2022; Chamizo et al. 2018, 2020; Chi et al. 2020; Giraldo-Silva et al. 2019; Kholssi et al. 2022; Rossi et al. 2017). The microbial biomass can improve soil structure and promote water retention, and stimulate plant growth by increasing the availability of nutrients or by releasing biologically active compounds (such as osmolytes, phenolics, proteins, vitamins, carbohydrates, amino acids, polysaccharides and phytohormones) that can act as natural biostimulants and or mitigate biotic and abiotic stresses (Alvarez et al. 2021; Kapoore et al. 2021; Ronga et al. 2019; Santini et al. 2021). Nevertheless, the interactions between these beneficial microorganisms and plants are still poorly understood and the mechanisms of action remain widely unknown.

The main goal of this work was to isolate and characterize several cyanobacteria and microalgae from biocrusts collected at Mortágua, Central Portugal, and to assess their potential as plant biostimulants. The best performing native microorganisms were tested as a consortium and insights into their mechanisms of action were obtained.

Materials and methods

Sampling, isolation and culture conditions

The cyanobacteria and microalgae isolated in this study were collected from soil biocrusts (Municipality of Mortágua, Viseu, Central Portugal) on April 16th, 2019, from two different sites: Sampling site 1—area surrounding a wood pellets factory (40°22′43.8"N; 8°11′13.1"W), and Sampling site 2—vacant land plot from the municipality (40°25′25.3"N; 8°11′20.3"W) (Fig. 1).

Soil biocrusts were observed using the Leica Zoom 2000 Stereo Microscope (Leica Microsystems, Wetzlar, Germany), detached from the soil, and inoculated into BG11 or $BG11_0$ medium (Stanier et al. 1971). For the isolation of cyanobacteria and microalgae from the initial mixed cultures, an aliquot was transferred to solid medium [BG11/$BG11_0$ supplemented with 1.5% (w/v) Difco® Agar Noble, 0.3% (w/v) sodium thiosulfate and 10 mM TES–KOH buffer (pH 8.2)], and the microorganisms isolated through successive spread plating and serial dilutions (Temraleeva et al. 2016). Cultures were observed under a light microscope throughout the isolation process and transferred to liquid medium when unicyanobacterial or unialgal cultures were obtained. Liquid cultures were kept at 25 ºC, under a 16 h light (15—25 µmol photons m^{-2} s^{-1})/8 h dark regimen, with or without agitation (200 rpm). The six microorganisms selected for consortium are deposited at LEGE Culture Collection, CIIMAR (Matosinhos, Portugal) under the identifications LEGE 191162 to 191166 (cyanobacteria) and LEGE 191161 M (microalga).

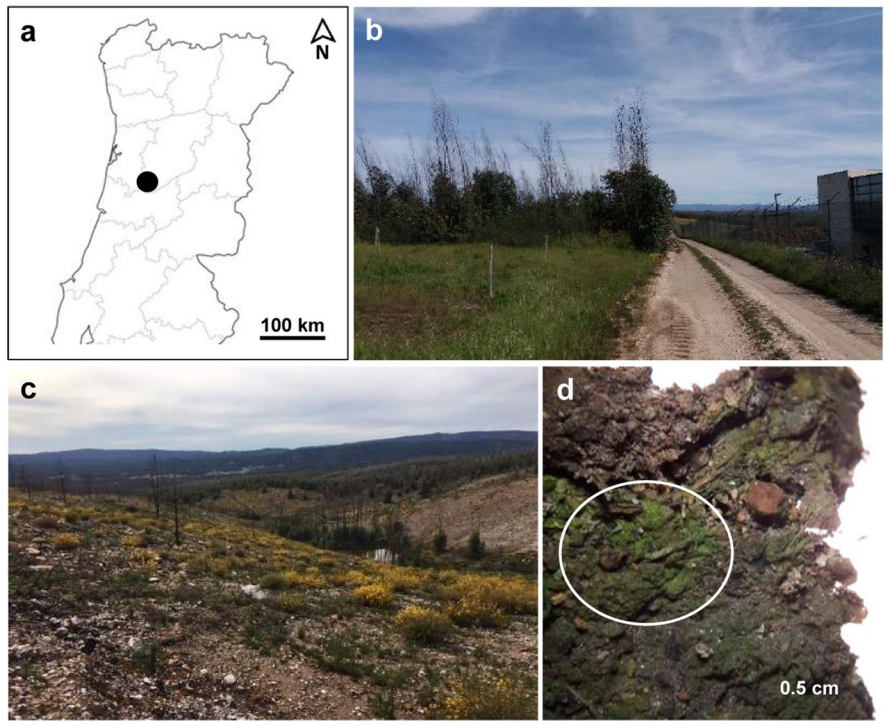


Fig. 1 Mortágua Municipality, Viseu, Central Portugal location (**a**), and view of the two sampling sites: Sampling site 1—area surrounding a wood pellets factory (**b**) and Sampling site 2—a vacant land plot from the municipality (**c**). Representative soil biocrust observed under the stereomicroscope (**d**)

Light microscopy and transmission electron microscopy (TEM)

Cells were observed directly using an Axio Lab.A1 light microscope (ZEISS, Oberkochen, Germany) and micrographs were acquired with an Axiocam ERc 5 s camera (ZEISS, Oberkochen, Germany) using the ZEN 2.6 software (ZEISS, Oberkochen, Germany).

For TEM, cells were collected by centrifugation and processed as previously described by Santos et al. (2021). Ultrathin sections were examined using a JEM-1400Plus (Jeol, MA, USA) electron microscope operating at 80 kV.

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted using the Plant/Fungi gDNA Isolation Kit (NYZtech, Lisbon, Portugal) according to the manufacturer's instructions. For PCR amplification of regions within the 16S rRNA gene, the internal transcribed spacer (ITS), the 23S rRNA gene (for cyanobacteria), or the 18S rRNA gene, ITS1-5.8S-ITS2, and 28S rRNA gene (for the microalga) the oligonucleotide primers listed in Table S1 were used. Each PCR reaction was performed in a final volume of 20 µL: 10 µL of Supreme NZYTaq II 2×Green Master Mix (NZYTech, Lisbon, Portugal), 1 µL of each primer (10 µM), and 5 -10 ng of DNA. The PCR profiles included an initial denaturation at 95 ºC for 5 min, followed by 35 cycles at 95 ºC for 1 min, 50 ºC for 1 min, 72 ºC for 1 min, and a final extension at 72 ºC for 7 min. PCR products were separated by agarose gel electrophoresis. DNA fragments were isolated from gels using the NZYGelpure Kit (NZYTech, Lisbon, Portugal), according to the manufacturer's instructions and sequenced at STAB Vida (Lisbon, Portugal). Each DNA fragment was sequenced at least three times to mitigate errors that could be introduced by the polymerase. Sequence data were deposited in the GenBank database under the accession numbers OK161231, OK161232, OK161253, OK161269, OK161270 (cyanobacteria) and OQ320396 (microalga).

Phylogenetic analysis

For the phylogenetic analysis, the cyanobacterial 16S rRNA and the microalgal 18S rRNA gene sequences were independently searched against the NCBI BLASTn database (October 2022) and their first 10 hits (ordered by the expect value),

were retrieved. In addition, selected sequences from relevant taxa, encompassing reference strains, were included in these analyses to obtain a reliable backbone representation of the cyanobacteria and microalgae diversity. Two phylogenetic trees based on the 16S rRNA gene (for the filamentous non-heterocystous and heterocystous cyanobacteria) and one based on the 18S rRNA gene (microalgae) were constructed using the following procedure: sequences were aligned using Clustal Omega (Sievers et al. 2011) and phylogenetic relationships were inferred using the Maximum-Likelihood (ML) and 1000 resamples [FastTree (Price et al. 2010)]. In FastTree, it was implemented the General Time Reversible model with a proportion of invariant sites and a gamma distribution (GTR + I + G). When using the Akaike Information Criterion (AIC), as implemented in jModeltest2 (Darriba et al. 2012), this was the best model for all three datasets, out of 24 candidate models. Using MEGA X (Kumar et al. 2018), the filamentous and heterocystous cyanobacteria phylogenies were rooted using *Gloeobacter violaceus* PCC 7421, while the microalgae phylogeny was rooted with *Chlorella vulgaris* and *Nephroselmis olivacea*. The Docker images available at the pegi3s Bioinformatics Docker Images Project for these programs (https://pegi3s.github.io/dockerfiles/; López-Fernández et al. 2022) were used to perform the analyses.

Detection of indole-3-acetic acid (IAA) and siderophores production

The IAA production was measured using the conditioned medium (microorganism exudate) from cells grown for about 4 weeks in standard conditions (see above) using the Salkowski's reagent (0.5 M FeCl3 in 35% perchloric acid) (Gordon and Weber 1951) and determined using a calibration curve made with an IAA standard (Duchefa biochemie) with concentrations from 0 to 10 µg mL^{-1}.

For siderophores detection the microorganisms were grown in BG11 medium without ferric ammonium citrate for 4 weeks at 25 ºC, under a 16 h light (15—25 µmol photons m^{-2} s^{-1})/8 h dark regimen, with agitation (70 rpm). The chrome azurol S (CAS) assay was performed using 300 µL of 10×concentrated conditioned medium (microorganism exudate). The plates were prepared using 30.24 g of piperazine-1,4-bis (2-ethanesulfonic acid) (PIPES), 72.9 mg of cetyltrimethylammonium bromide (CTAB), 1 mM of $FeCl_3.6H_2O$ in 10 mM HCl, 60.5 mg of CAS, and 1% (w/v) agarose as gelling agent per L (Schwyn and Neilands 1987).

Petri dish plant cultures

Liquid cultures of each isolated microorganism (one month old) were centrifuged for 2 min at 10,000 g, and 2 mL of the supernatants were spread into square sterile Petri dishes containing solid Hoagland medium (Sigma), with 1.6% (w/v) agar (Labkem), or solid Hoagland medium supplemented with NaCl (100 mM or 125 mM). For the controls 2 mL of BG11 medium (Stanier et al. 1971) were used.

Seeds of *Lolium multiflorum* cv. Diamond T. (Ore-Gro Seeds Inc.) and *Arabidopsis thaliana* ecotype Col-0 (Nottingham Arabidopsis Stock Center) were superficially sterilized with 10% (v/v) commercial bleach, washed and soaked in distilled water, and germinated in the previously prepared Petri dishes. This experiment had two controls: plants grown on solid Hoagland medium (control) and plants grown on solid Hoagland medium with NaCl (control + NaCl). The plants growth occurred under controlled conditions [16 h light (100 µmol m^{-2} s^{-1}) / 8 h dark at 23 ± 2 °C]. After 10 (*L. multiflorum*) or 21 (*A. thaliana*) days, the plant biometric parameters (number of leaves, root length and fresh weight) were evaluated.

Hydroponic plant cultures

The seedlings of *L. multiflorum* and *A. thaliana* seeds grown for 10 or 21 days, respectively, in Petri dishes with Hoagland medium supplemented with 1.6% (w/v) agar, were detached and transferred to hydroponic systems (Hydro 60, GroHo). Initially the seedlings were covered with cling film that was progressively removed after 3—7 days. Ten liters of Hoagland solution were used to fill each of the growth medium reservoir of the hydroponic systems and, in one of the systems, the Hoagland solution was supplemented with the microorganism's consortium (0.6 mg of chlorophyll *a*). Every week, the ten liters volume was adjusted with Hoagland solution to counterbalance the evaporation. Each of the consortium microorganism was grown independently for at least one month and the consortium was established

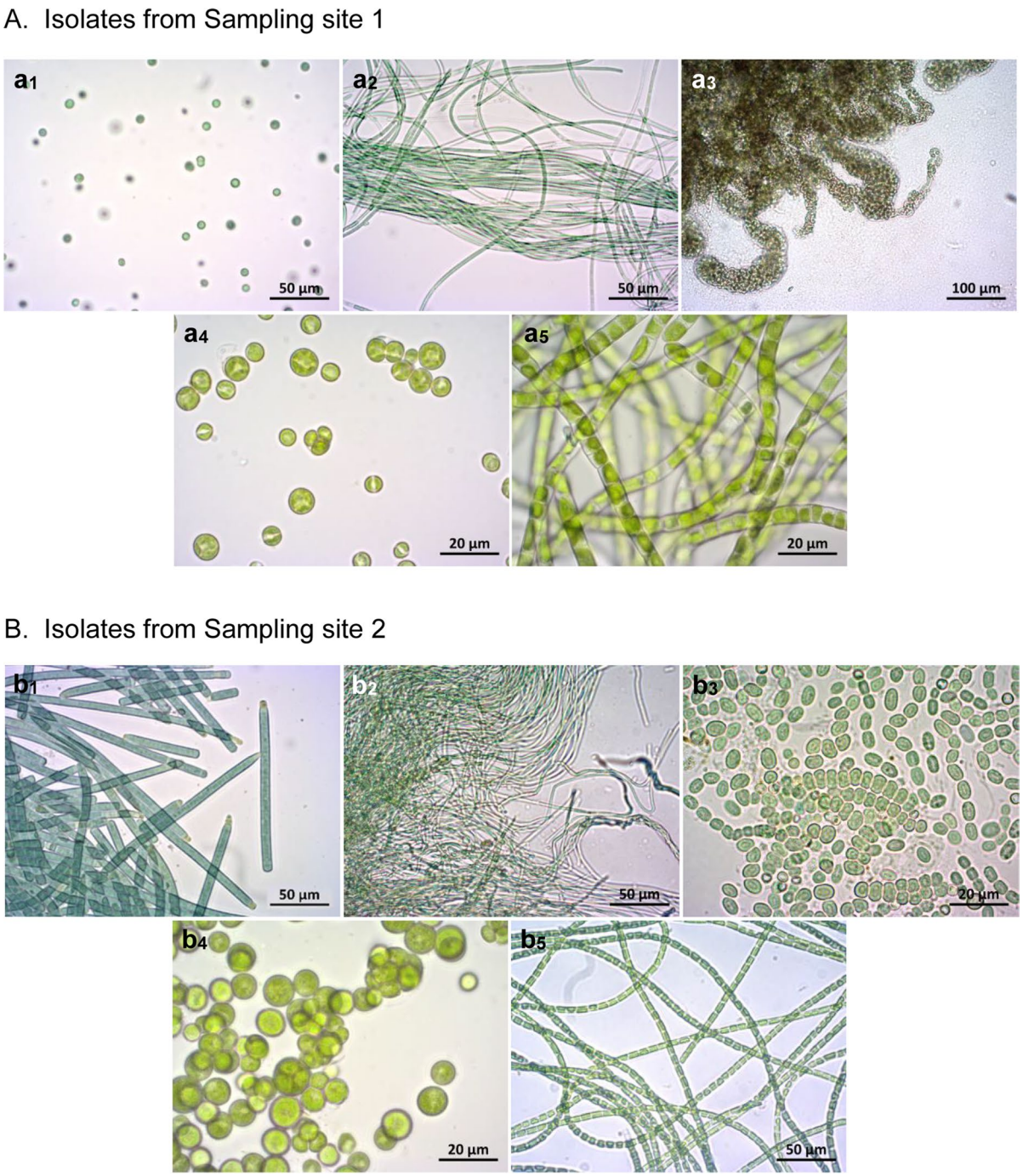
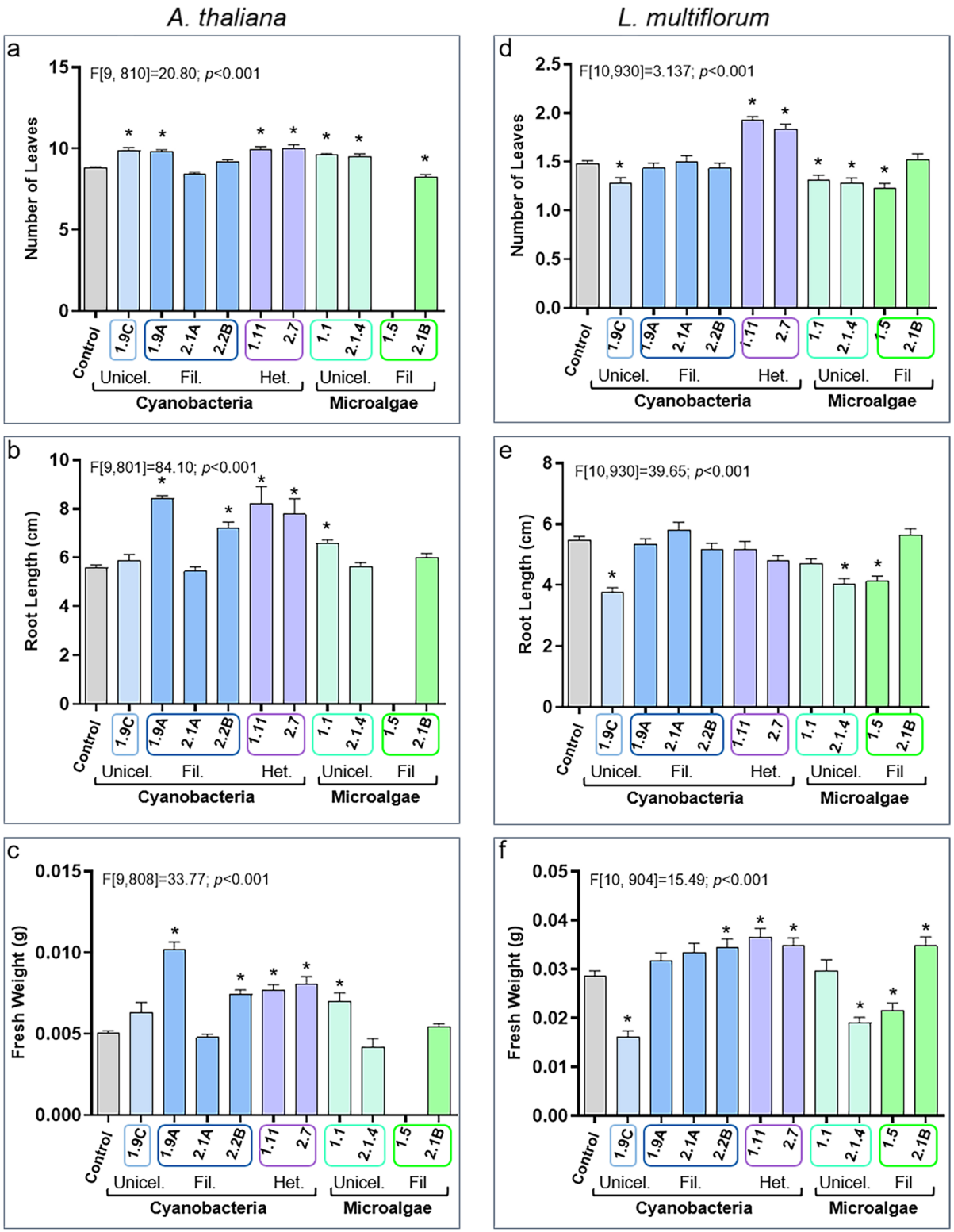


Fig. 2 Light micrographs of the cyanobacteria and microalgae isolated from soil biocrusts collected from Sampling site 1 (**a**) and Sampling site 2 (**b**) (for details see Fig. 1). **Sampling site 1**: Unicellular cyanobacterium: isolate 1.9C ($\mathbf{a_1}$), filamentous cyanobacterium: isolate 1.9A ($\mathbf{a_2}$), heterocystous cyanobacterium: isolate 1.11 ($\mathbf{a_3}$), unicellular and filamentous microalgae: isolate 1.1 ($\mathbf{a_4}$) and 1.5 ($\mathbf{a_5}$), respectively. **Sampling site 2**: Filamentous cyanobacteria: isolate 2.1A ($\mathbf{b_1}$) and 2.2B ($\mathbf{b_2}$), heterocystous cyanobacterium: isolate 2.7 ($\mathbf{b_3}$), unicellular and filamentous microalgae: isolate 2.1.4 ($\mathbf{b_4}$) and 2.1B ($\mathbf{b_5}$), respectively


A. thaliana
L. multiflorum
a
F[9, 810]=20.80; p<0.001
Number of Leaves
b
F[9,801]=84.10; p<0.001
Root Length (cm)
c
F[9,808]=33.77; p<0.001
Fresh Weight (g)
d
F[10,930]=3.137; p<0.001
Number of Leaves
e
F[10,930]=39.65; p<0.001
Root Length (cm)
f
F[10, 904]=15.49; p<0.001
Fresh Weight (g)
Control
1.9C
1.9A
2.1A
2.2B
1.11
2.7
1.1
2.1.4
1.5
2.1B
Unicel.
Fil.
Het.
Unicel.
Fil
Cyanobacteria
Microalgae

◂**Fig. 3** Effect of the microorganisms' exudates on *Arabidopsis thaliana* and *Lolium multiflorum* growth under standard conditions. *A. thaliana* (**a**, **b**, **c**) and *L. multiflorum* (**d**, **e**, **f**) plantlets were grown in Petri dishes with Hoagland medium supplemented with the respective microorganism exudate (except for the control). The number of leaves (**a**, **d**), root length (**b**, **e**) and fresh weight (**c**, **f**) were evaluated. The results are expressed as means ± SE. One-way ANOVA tests were performed with Dunnett´s multiple comparison, comparing all datasets to the control. * indicates statistically significant differences ($p < 0.05$). Unicel. – unicellular: Fil. –filamentous; Het. – heterocystous

just to the application in the hydroponic culture. The plants were grown for 2 months under environmental conditions in a greenhouse during spring [12 h light (25 ± 2 °C)/ 12 h dark (18 ± 2 °C), and subsequently the plant biometric parameters (number of leaves, root length and root and shoot fresh weight) were evaluated. The plant material was preserved using liquid nitrogen and stored at -80 ºC for biochemical analysis.

pH and electrical conductivity measurements

pH and electrical conductivity were measured using a Consort 3030 Multiparameter analyzer.

Plant biochemical determinations

Proline, glutathione, H_2O_2 and lipid peroxidation levels, as well as the activity of the enzymes Glutamine Synthetase (GS), Catalase (CAT) and Ascorbate Peroxidase (APX), were quantified as previously described (Brito et al. 2022).

For the quantification of chlorophyll *a* (Chl *a*) and *b* (Chl *b*) 200 mg of plant material were macerated in 8 mL of 80% (v/v) acetone and centrifuged for 10 min at 4000 g, at room temperature and protected from light. The absorbance of the supernatant was measured at 647 nm and 663 nm and the chlorophyll concentration calculated according to the equations provided by Lichtenthaler and Buschmann (2001).

For the quantification of starch and soluble sugars 40 mg of plant material were homogenized in 5 mL of ethanol 80% and left at 80 ºC for 1 h. After vortex and centrifugation at 5000 g, for 10 min at 4 ºC, both pellet and supernatant were collected. The supernatant was used for the quantification of the soluble sugar contents by reaction with anthrone in accordance to the protocol described in Irigoyen et al. (1992), adapted to microplates. The pellet was resuspended in 3 mL of perchloric acid 30%, incubated at 60 ºC for 1 h, centrifuged at 10,000 g for 10 min at 4 ºC and the supernatant was used for the quantification of the starch content by reaction with anthrone following the protocol provided by Osaki et al. (1991), adapted to microplates.

Statistical analysis

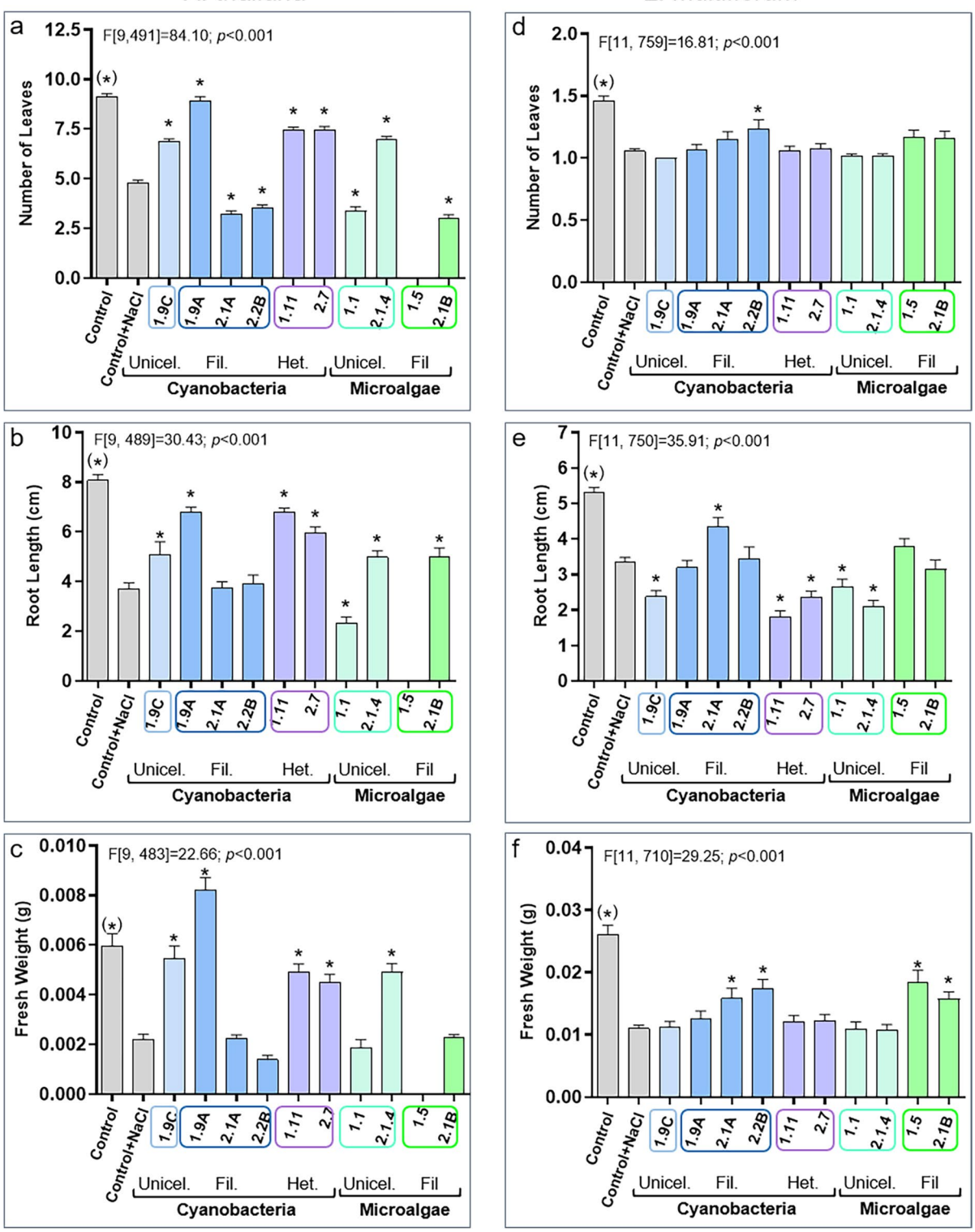
For the Petri dish experiments at least three independent assays, using three dishes with 10–20 plantlets, were performed. For the hydroponic experiments three independent assays were conducted using 10–20 plants each. For the biochemical quantification each hydroponic experiment was treated as a pool, and at least 3 independent technical replicates were made, with the results expressed as mean ± standard error (SE). The results were tested for normality by the Shapiro–Wilk test and homogeneity of variances by Levene's test, and then the comparisons between the treatments and the control were made using two types of tests: unpaired t-test with Welch´s correction ($p < 0.05$) for analysis of only two datasets and One-Way ANOVA, followed by Dunnett´s multiple comparison test ($p < 0.05$), for comparing several datasets to the control group, both performed using the GraphPad® Prism 7 software (GraphPad Software Inc., USA). A significance level (α) of 0.05 was used in all analyses.

Results

Isolation of cyanobacteria and microalgae from soil biocrusts

Soil biocrusts samples were collected in the Municipality of Mortágua (Viseu, Central Portugal, for details see Fig. 1, Materials and methods) and several cyanobacterial strains and microalgae species were isolated leading to six unicyanobacterial cultures (1 unicellular, 3 filamentous and 2 heterocystous) and four unialgal cultures (2 unicellular and 2 filamentous) (Fig. 2).

Since it is well documented that soil microorganisms can produce bioactive substances that promote plant growth and/or tolerance to abiotic stresses


A. thaliana
L. multiflorum
a
F[9,491]=84.10; p<0.001
Number of Leaves
b
F[9, 489]=30.43; p<0.001
Root Length (cm)
c
F[9, 483]=22.66; p<0.001
Fresh Weight (g)
d
F[11, 759]=16.81; p<0.001
Number of Leaves
e
F[11, 750]=35.91; p<0.001
Root Length (cm)
f
F[11, 710]=29.25; p<0.001
Fresh Weight (g)
Control
Control+NaCl
1.9C
1.9A
2.1A
2.2B
1.11
2.7
1.1
2.1.4
1.5
2.1B
Unicel.
Fil.
Het.
Unicel.
Fil
Cyanobacteria
Microalgae

◂**Fig. 4** Effect of the microorganisms' exudates on *Arabidopsis thaliana* and *Lolium multiflorum* growth under high salinity conditions. *A. thaliana* (**a**, **b**, **c**) and *L. multiflorum* (**d**, **e**, **f**) plantlets were grown in Petri dishes with Hoagland medium supplemented with the respective microorganism exudate (except for the control) and in the presence of 125 mM (*A. thaliana*) or 100 mM (*L. multiflorum*) NaCl. The number of leaves (**a**, **d**), root length (**b**,**e**) and fresh weight (**c**,**f**) were evaluated. The results are expressed as means ± SE. One-way ANOVA tests were performed with Dunnett´s multiple comparison, comparing all datasets to the control with NaCl (Control + NaCl). * indicates statistically significant differences ($p < 0.05$). Unicel. – unicellular: Fil. – filamentous; Het. – heterocystous

(Kapoore et al. 2021; Santini et al. 2021), an initial experiment using the exudates (conditioned medium) from our isolates was performed.

Effect of the microorganisms' exudates on plant growth

To evaluate the effect of the microorganisms' exudates on plant growth, seeds of two plant species, the dicotyledonous *Arabidopsis thaliana* and the monocotyledonous *Lolium multiflorum*, were germinated in Petri dishes containing nutritive medium supplemented with each of the microorganism's exudates. Moreover, to understand if the microorganisms' exudates have a protective effect under high salinity conditions the plants were grown with medium supplemented with NaCl (100 mM for *L. multiflorum* and 125 mM for *A. thaliana*, considering the species sensibility). The number of leaves, root length and fresh weight of the plantlets were evaluated (Figs. 3 and 4).

Under standard conditions, most of the microorganisms' exudates had positive effects or did not affect *A. thaliana* growth, except isolate 1.5 that causes plant death after 10 days. Five microorganisms stood out as enhancers of most of the biometric parameters, namely the filamentous cyanobacteria 1.9A and 2.2B, the heterocystous cyanobacteria 1.11, 2.7, and the microalga 1.1. Regarding *L. multiflorum* the filamentous cyanobacterium 2.2B, the two heterocystous cyanobacteria (1.11, 2.7) and the filamentous microalga 2.1B had positive effects, while the unicellular cyanobacterium 1.9C, the unicellular microalga 2.1.4, and the filamentous microalga 1.5 had negative effects in most of the biometric parameters evaluated.

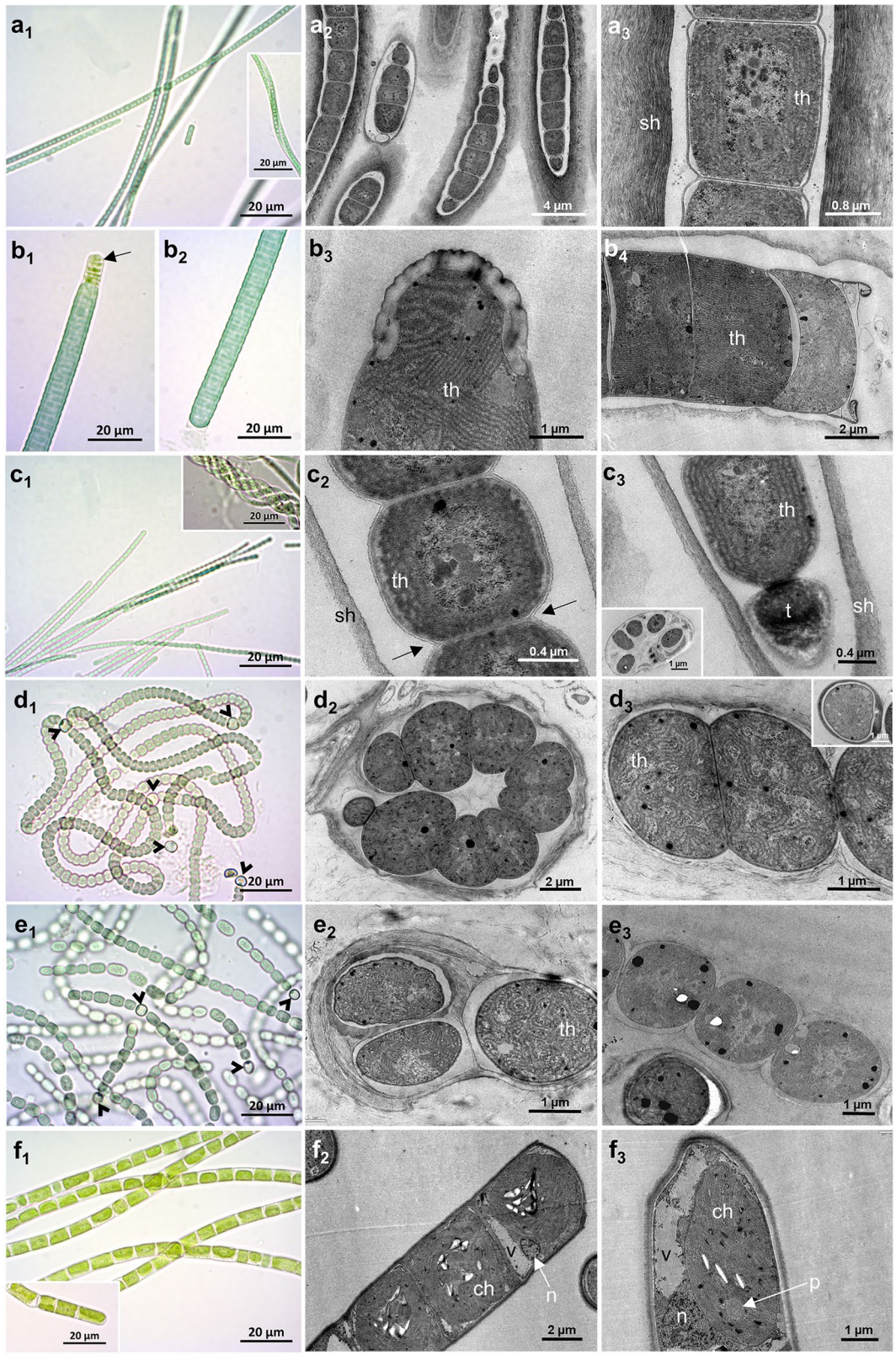
Under high salinity conditions, the impairment of *A. thaliana* growth caused by the salt stress was attenuated in the presence of several exudates, namely from the unicellular cyanobacterium 1.9C, the filamentous 1.9A, the heterocystous 1.11 and 2.7, as well as from the unicellular microalga 2.1.4. Regarding *L. multiflorum*, the filamentous cyanobacteria 2.1A and 2.2B and the microalgae 1.5 and 2.1B reduced the growth impairment in some of the parameters assessed.

Considering the Petri dish experiment results, the microorganisms with a widespread negative effect on plant growth were excluded, namely the microalga 1.5 as result of a lethal effect on *A. thaliana*, and the unicellular cyanobacterium 1.9C and the unicellular microalgae 1.1 and 2.1.4, due to their negative effects on *L. multiflorum*. Considering together the results obtained here and in a previous study (Gonçalves et al. 2022), the other six microorganisms were selected for subsequent studies: The filamentous cyanobacterium 1.9A due to the extensive positive effects on *A. thaliana* growth and protection against salinity stress, as well as for a clear effect on soil rehabilitation (Gonçalves et al. 2022); The two heterocystous cyanobacteria (1.11 and 2.7) for improving growth of the two-plant species under standard conditions, and for protecting *A. thaliana* from salt stress; The two filamentous cyanobacteria, 2.1A and 2.2B and the filamentous microalga 2.1B, because they did not show any deleterious effect on the plants' growth and two of them even stimulated soil rehabilitation (Gonçalves et al. 2022).

Characterization of the six microorganisms selected

Filamentous non-heterocystous cyanobacteria

Isolate 1.9A exhibits trichomes slightly curved or entangled together (Fig. 5a1) with highly variable cell lengths typically longer than wide or isodiametric (Fig. 5a2). The sheath is clearly visible in the transmission electron micrographs, as well as the parietal arrangement of the thylakoids (Fig. 5a3). Regarding the molecular analyses, a sequence comprising most of the 16S-ITS-23S rRNA region was obtained and used to perform a BLASTn search using the NCBI database (October 2022), showing 97.68% similarity to *Trichocoleus desertorum* SBC54 (MW403955.1). In addition,


a1
20 µm
20 µm
a2
4 µm
a3
sh
th
0.8 µm
b1
20 µm
b2
20 µm
b3
th
1 µm
b4
th
2 µm
c1
20 µm
20 µm
c2
th
sh
0.4 µm
c3
th
t
sh
1 µm
0.4 µm
d1
20 µm
d2
2 µm
d3
th
1 µm
1 µm
e1
20 µm
e2
th
1 µm
e3
1 µm
f1
20 µm
20 µm
f2
v
ch
n
2 µm
f3
ch
v
n
p
1 µm

◂**Fig. 5** Light and electron transmission micrographs of the six microorganisms selected for the consortium: filamentous cyanobacteria (**a**-**c**), heterocystous cyanobacteria (**d**, **e**), and filamentous microalga (**f**). Filaments of *Trichocoleus* sp. (isolate 1.9A) entangled together ($\mathbf{a_1}$, insert) and its ultrastructure showing the parietal arrangement of the thylakoids (th) and the sheath (sh) ($\mathbf{a_2}$,$\mathbf{a_3}$). *Microcoleus* sp. (isolate 2.1A) where it is possible to observe the extremities of the trichomes with the distinct morphology at the ends ("head" and "tail") ($\mathbf{b_1}$,$\mathbf{b_2}$) and TEM micrographs ($\mathbf{b_3}$,$\mathbf{b_4}$) where is evident the irregular arrangement of the thylakoids (th). *Nodosilinea* sp. (isolate 2.2B) ($\mathbf{c_1}$) with the characteristic spirals formed by twisted filaments (insert). The deeply constricted cross-walls (arrows), the parietal arrangement of the thylakoids (th), the sheath (sh), the conical shape of the apical cell (t), and a nodule detail (insert) can clearly be observed in the TEM micrographs ($\mathbf{c_2}$,$\mathbf{c_3}$). *Nostoc* sp. (isolate 1.11) with the heterocysts indicated by arrow heads ($\mathbf{d_1}$). The coiled filaments are surrounded by an extracellular polysaccharidic matrix, the thylakoids (th) have an irregular arrangement, and the heterocysts can be intercalated or terminal (insert) ($\mathbf{d_2}$,$\mathbf{d_3}$). *Nostoc* sp. (isolate 2.7) with heterocysts indicated by arrow heads ($\mathbf{e_1}$) and TEM micrographs ($\mathbf{e_2}$,$\mathbf{e_3}$) showing trichomes embedded in a mucilaginous material, as well as the irregular arrangement of the thylakoids (th). Microalga *Klebsormidium* sp. (isolate 2.1B) where it is possible to observe the chloroplasts along the filaments' cells ($\mathbf{f_1}$). TEM micrographs ($\mathbf{f_2}$,$\mathbf{f_3}$) showing chloroplasts (ch), pyrenoid (p), nuclei (n), and vacuoles (v)

the phylogenetic analysis, based on 16S rRNA gene sequences, showed that isolate 1.9A was positioned within a coherent cluster comprising *Trichocoleus* strains supported by a strong bootstrap value (Fig. 6).

Isolate 2.1A exhibits straight or slightly curved trichomes with a distinct morphology at the ends (Fig. 2b1) with one extremity displaying an apical rounded cell while the other capitate end is yellowish with several adjacent cells (Fig. 5b1-b4). The TEM revealed that the thylakoids form fascicle-like aggregations arranged irregularly (Fig. 5b3,b4), and that the disk-shaped cells are consistently wider than long with the formation of new cell walls perpendicularly to the trichome axis (Fig. 5b4). The BLASTn search showed the 16S-ITS-23S rRNA region sequence for this isolate has 97.82% similarity to *Microcoleus vaginatus* HSN003 (CP031705.1). In the phylogenetic analysis, isolate 2.1A was placed within a cluster, containing mainly *Microcoleus* strains, with a strong bootstrap support (Fig. 6).

Isolate 2.2B exhibits trichomes with different curvatures (straight, tangled together or twisted forming spirals) (Figs. 2b2 and 5c1), cells are usually longer than wide or isodiametric, and barrel shaped with sharp constrictions at the cells' junctions (Fig. 5c2,c3). The trichomes are surrounded by a colorless sheath (Fig. 5c1-c3). TEM micrographs revealed the parietal arrangement of the thylakoids, the conical shape of the apical cells, and the occurrence of nodules (short and convoluted trichomes within a bounding sheath) (Fig. 5c3 and insert). The BLASTn search analysis showed the 16S-ITS-23S rDNA sequence obtained has 97.16% similarity to *Nodosilinea* cf. *signiensis* NN-3–1-CD (MT946555.1). In the phylogenetic analysis the isolate 2.2B is within a cluster with a strong bootstrap support, mostly composed by *Nodosilinea* strains (Fig. 6), including the type species *N. nodulosa* UTEX 2910 (Perkerson et al. 2011).

Heterocystous cyanobacteria

Isolate 1.11 forms gelatinous colonies, with deeply coiled trichomes surrounded by an extracellular polysaccharidic matrix (Figs. 2a3 and 5d2). The heterocysts are spheric to oval and were observed at intercalary and terminal positions (Fig. 5d1,d3 and insert). The rounded vegetative cells are constricted at the cells' junctions and the thylakoids have a fascicular arrangement with spherical formations (Fig. 5d2,d3). The sequence comprising the majority of the 16S-ITS rRNA region and used to perform a BLASTn search showed 96.08% similarity to *Nostoc punctiforme* PCC 73102 (CP001037.1). The phylogeny placed the isolate 1.11 within a coherent cluster, with a strong bootstrap value, containing *Nostoc* strains only, including the type species *N. commune* EV1 KK1 (AY577536) (Fig. 7).

Isolate 2.7 displays vegetative cells with highly variable shape/lengths (Fig. 2b3 and Fig. 5e1) and more or less spherical heterocysts in intercalary and terminal positions (Fig. 5e1). TEM micrographs showed trichomes embedded in an extracellular polysaccharidic matrix (Fig. 5e2) and fascicular arrangement of the thylakoids with spherical formations (Fig. 5d2,d3). The 16S-ITS rRNA region sequence showed 96.18% similarity to *Nostoc* cf. *commune* SO-36 (AP025732.1). In the phylogenetic analysis, the isolate 2.7 is within a cluster comprising several *Nostoc* strains but not only (Fig. 7).

Filamentous microalga

Isolate 2.1B exhibits long unbranched uniseriated filaments without the tendency to break and short to elongated cylindrical cells (Figs. 2b5 and 5f1) with a single parietal chloroplast encircling half to 2/3 of

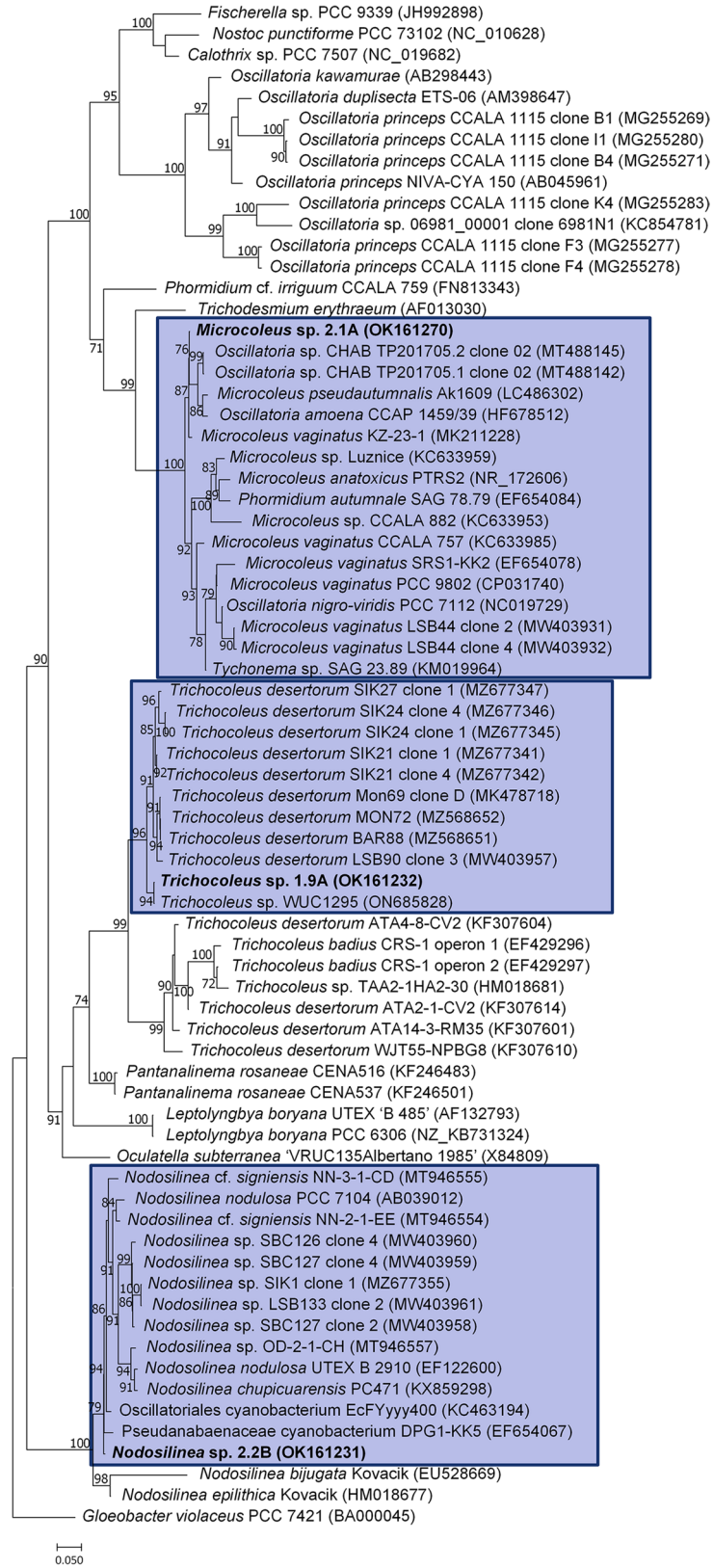


Fig. 6 Maximum likelihood phylogenetic tree based on partial 16S rRNA gene sequences from cyanobacteria. The three filamentous non-heterocystous strains isolated in this study from biocrusts collected at Mortágua, Central Portugal (*Microcoleus* sp. 2.1A, *Trichocoleus* sp. 1.9A, and *Nodosilinea* sp. 2.2B) are indicated in bold and by the respective GenBank accession number (clades highlighted in blue). *Gloeobacter violaceus* PCC 7421 was used as outgroup. Numbers along branches indicate bootstrap values considering 1000 pseudo-replicates: only those equal or above 70% are shown

Fig. 7 Maximum likelihood phylogenetic tree based on partial 16S rRNA gene sequences from filamentous heterocystous cyanobacteria. The two strains isolated in this study *(Nostoc* sp. 1.11 and *Nostoc* sp. 2.7) are indicated in bold and by the respective GenBank accession number (clades highlighted in purple). *Gloeobacter violaceus* PCC 7421 was used as outgroup. Numbers along branches indicate bootstrap values considering 1000 pseudo-replicates: only those equal or above 70% are shown

the cell wall and varying the side in each cell. The chloroplasts (Fig. 5f1) contain rounded pyrenoids and a variable number of starch grains (Fig. 5f2,f3). The sequence obtained for the 18S rRNA gene showed 99.45% similarity to *Klebsormidium dissectum* SAG 2155 (EF372518.1). The phylogenetic analysis placed isolate 2.1B within a cluster composed by *Klebsormidium* strains only, including the type species *K. flaccidum*, with a bootstrap support of 96% (Fig. 8).

Production of indole-3-acetic acid (IAA) and siderophores by the selected microorganisms

In order to gain an insight into mechanisms that may be involved in the beneficial interaction between the microorganisms and the plants, the production of the phytohormone IAA and the capacity to release siderophores was determined.

One of the filamentous and one of the heterocystous cyanobacterial strains, *Trichocoleus* sp. (isolate

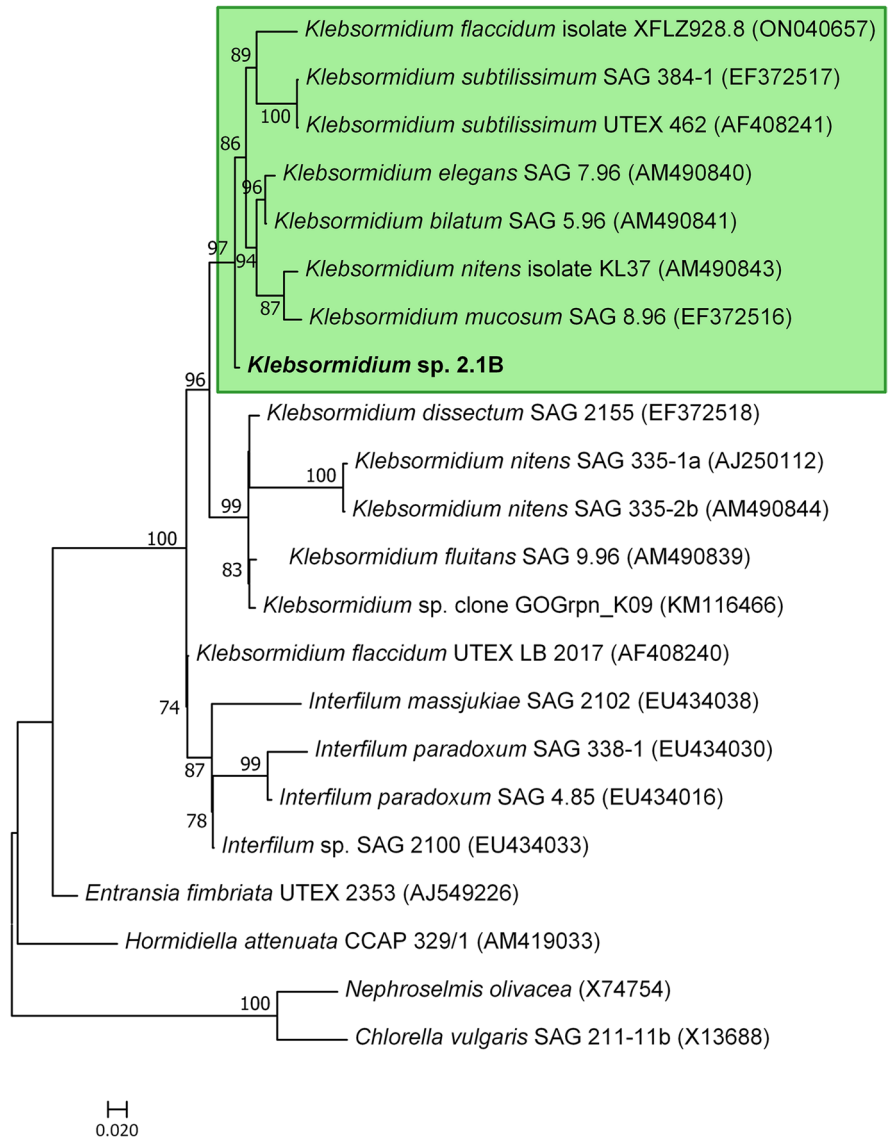


Fig. 8 Maximum likelihood phylogenetic tree based on partial 18S rRNA gene sequences from microalga. The filamentous microalga *Klebsormidium* sp. 2.1B isolated in this study is indicated in bold and by the respective GenBank accession number (clade highlighted in green). *Nephroselmis olivacea* and *Chlorella vulgaris* were used as outgroup. Numbers along branches indicate bootstrap values considering 1000 pseudo-replicates: only those equal or above 70% are shown

1.9A) and *Nostoc* sp. (isolate 1.11), secrete IAA (Table 1), while both diazotrophic heterocystous cyanobacteria were able to release siderophores under iron limiting conditions (Fig. 9).

Table 1 Production of indole-3-acetic acid (IAA)

		Isolate	Organism	IAA µg mL^{-1}
Cyanobacteria	Filamentous	1.9A	*Trichocoleus* sp.	0.6
		2.1A	*Microcoleus* sp.	n.d.
		2.2B	*Nodosilinea* sp.	n.d.
	Heterocystous	1.11	*Nostoc* sp.	1.3
		2.7	*Nostoc* sp.	n.d.
Microalga	Filamentous	2.1B	*Klebsormidium* sp.	n.d.

n.d. not detectable

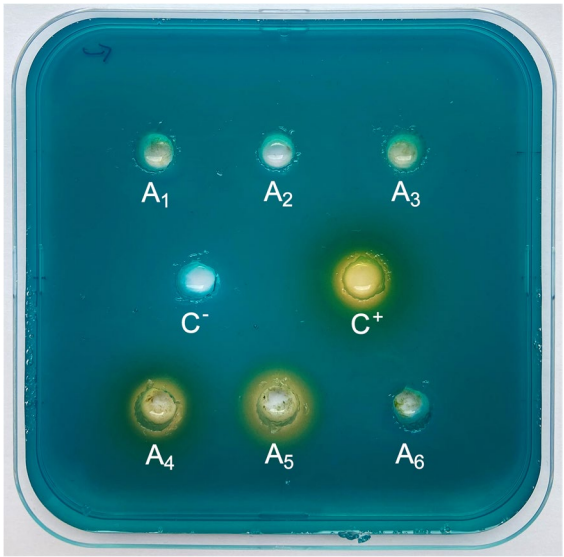


Fig. 9 Detection of siderophores production using the Chrome Azurol S (CAS) assay. **A_1:** *Trichocoleus* sp. (isolate 1.9A); **A_2:** *Microcoleus* sp. (isolate 2.1); **A_3:** *Nodosilinea* sp. (isolate 2.2B); **A_4:** *Nostoc* sp. (isolate 1.11); **A_5:** *Nostoc* sp. (isolate 2.7); **A_6:** *Klebsormidium* sp. (isolate 2.1B). The microorganisms were grown in BG11 medium without ammonium ferric citrate. 300 µL of 10×concentrated medium (microorganism exudate) was placed in each well. **C^-:** Negative control, 10×concentrated BG11 medium, **C^+:** Positive control, 300 µL of 2x concentrated medium from *Streptomyces tsukubaensis* Δ*sigG* grown in iron limited conditions (Oliveira et al. 2020)

Effect of the selected microorganisms' as consortium on plant growth

The six microorganisms previously selected and characterized were grown independently and then combined according to Table 2 to be tested as a consortium on plant growth. For this purpose, *A. thaliana* and *L. multiflorum* were grown in hydroponic systems, for one month, with nutritive medium (control) or nutritive medium supplemented with the microorganisms' consortium and several biometric and biochemical parameters were evaluated.

As it can be observed in Fig. 10, the consortium microorganisms promote both plant species growth. While for *A. thaliana* the consortium significantly increased the root length and shoot fresh weight, for *L. multiflorum* a significant increase in all the parameters evaluated (number of leaves, root length, root and shoot fresh weight) was noticed.

To gain insight into the microorganisms' consortium mechanism of action, some plants' biochemical parameters were analyzed, namely antioxidant system and productivity related markers. Concerning the antioxidant system, and as it can be observed in Fig. 11, the lipid peroxidation and H_2O_2 content of the leaves, did not change in the presence of the microorganisms for both plant species (Fig. 11a1,a2,b1,b2). The proline content increased with the presence of the consortium, while a decrease in the activity of catalase and ascorbate peroxidase was also observed for both species (Fig. 11c1,c2,d1,d2,e1,e2). Regarding the productivity related parameters (Fig. 12), the soluble sugars content and the activity of the enzyme glutamine synthetase increased in *A. thaliana* (Fig. 12b1,c1), while the levels of chlorophyll increased in *L. multiflorum* in the presence of the consortium (Fig. 12a2).

In order to understand if there was a change on the osmolarity of the hydroponic solution due to the presence of the microorganisms' consortium, the pH and electrical conductivity were measured. Only a minor difference in the electrical conductivity was detected in the presence of the consortium (2,47 mS cm^{-1})

Table 2 Microorganisms' consortium

		Isolate	Organism	LEGE* ID	Chlorophyll *a* ratio (%)
Cyanobacteria	Filamentous	1.9A	*Trichocoleus* sp.	191166	22.5
		2.1A	*Microcoleus* sp.	191162	22.5
		2.2B	*Nodosilinea* sp.	191163	22.5
	Heterocystous	1.11	*Nostoc* sp.	191164	5.0
		2.7	*Nostoc* sp.	191165	5.0
Microalga	Filamentous	2.1B	*Klebsormidium* sp.	191161 M	22.5

*LEGE Culture Collection (CIIMAR, Matosinhos, Portugal)

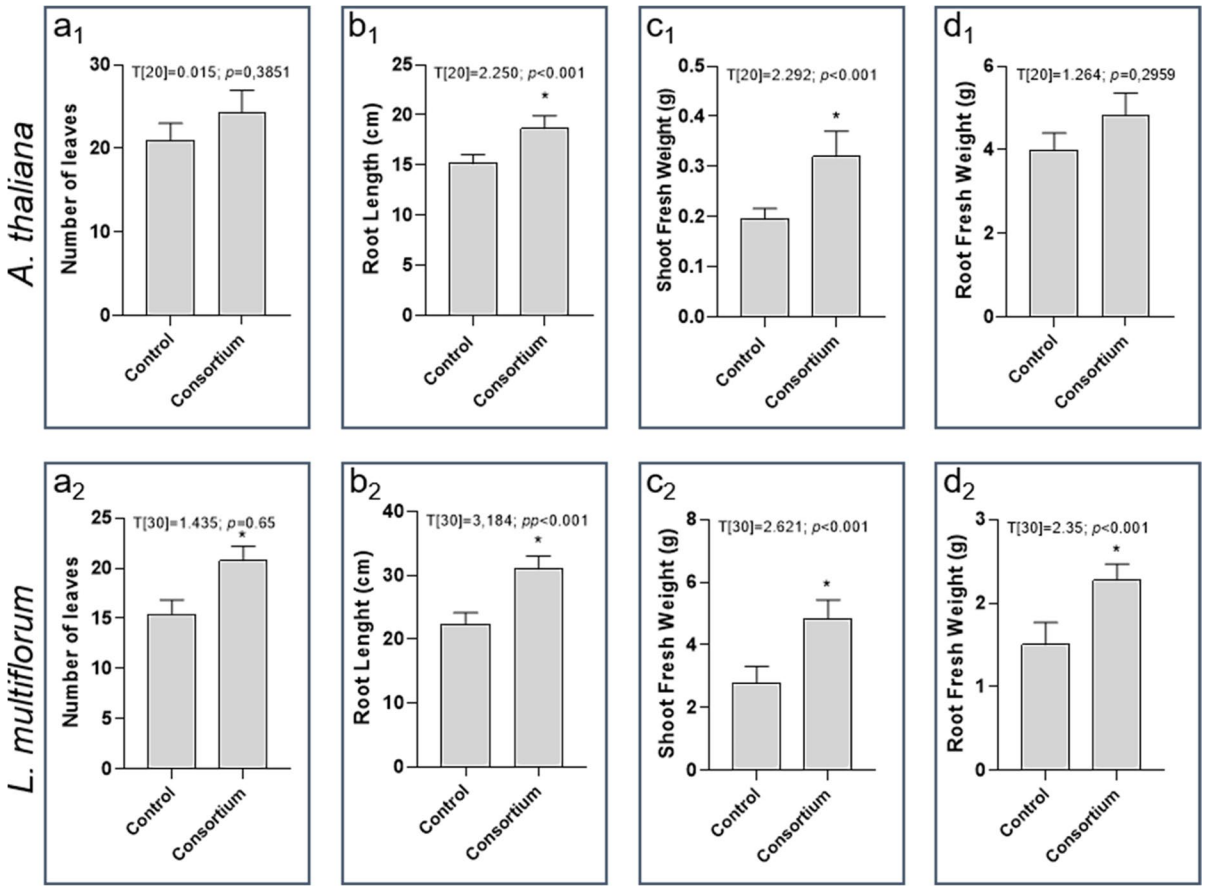


Fig. 10 Effect of the microorganisms' consortium on *Arabidopsis thaliana* and *Lolium multiflorum* growth. *A. thaliana* ($\mathbf{a_1}$-$\mathbf{d_1}$) and *L. multiflorum* ($\mathbf{a_2}$-$\mathbf{d_2}$) plants were grown in hydroponic cultures with Hoagland medium (Control) or Hoagland medium supplemented with the microorganisms' consortium (Consortium) (for details see Table 2). The number of leaves ($\mathbf{a_1}$,$\mathbf{a_2}$), root length ($\mathbf{b_1}$,$\mathbf{b_2}$), and shoot ($\mathbf{c_1}$,$\mathbf{c_2}$) and root ($\mathbf{d_1}$,$\mathbf{d_2}$) fresh weight were evaluated. The results are expressed as means ± SE. Unpaired Student's t-tests, with Welch's correction, were performed, comparing treated plants to the control. * indicates statistically significant differences ($p < 0.05$)

compared to the control (2,38 mS cm^{-1}) after a week (before the weekly refill of the Hoagland solution).

Discussion

Soil microbiota is pivotal for the maintenance of soil structure, properties and functionality. Cyanobacteria and microalgae are primary producers ubiquitously present in soils and pioneers in the establishment of the biocrusts (see for e.g. Chamizo et al. 2016; Ferrenberg et al. 2017; Rossi et al. 2017; Weber et al. 2022), but their biodiversity and characteristics remain largely understudied compared to their aquatic counterparts.

Here, several cyanobacteria and microalgae were isolated from biocrusts collected at Mortágua (Central Portugal), a region severely devastated by wildfires in 2017. Thus, this work constitutes a stepping stone into the development of a system for the rehabilitation of burned soils based on the inoculation of native photosynthetic microorganisms (GreenRehab project: www.greenrehab.pt). To establish a consortium that would benefit plant growth and/or improve soil characteristics, an initial screening in Petri dishes was performed using exudates from the isolated microorganisms and two plant species native of the Mediterranean flora, *Arabidopsis thaliana* and *Lolium multiflorum*.

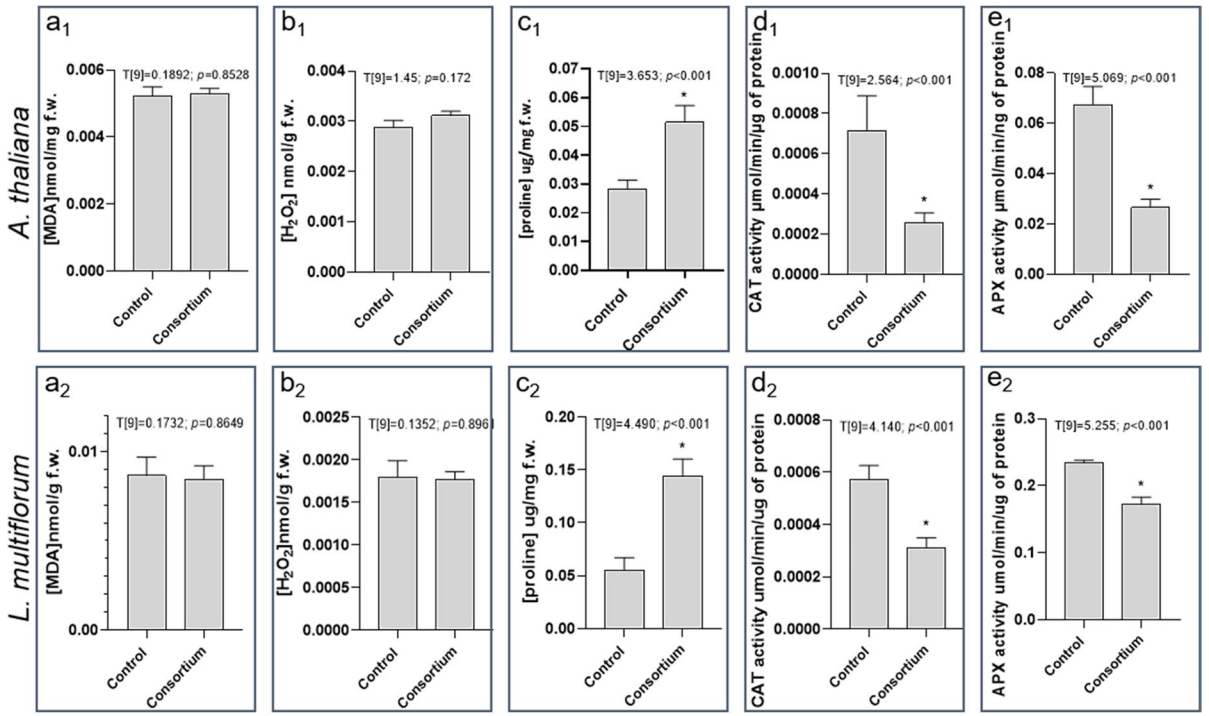


Fig. 11 Effect of the microorganisms' consortium on *Arabidopsis thaliana* and *Lolium multiflorum* antioxidant system. *A. thaliana* (**a_1**-**e_1**) and *L. multiflorum* (**a_2**-**e_2**) plants were grown in hydroponic cultures with Hoagland medium (Control) or Hoagland medium supplemented with the microorganisms' consortium (Consortium) (for details see Table 2). The lipid peroxidation expressed by the level of malondialdeyde – MDA (**a_1**,**a_2**), the H_2O_2 content (**b_1**,**b_2**), the proline content (**c_1**,**c_2**), and the enzymatic activities of catalase – CAT (**d_1**,**d_2**) and ascorbate peroxidase – APX (**e_1**,**e_2**) were evaluated. The results are expressed as means ± SE. Unpaired Student's t-tests, with Welch's correction, were performed, comparing treated plants to the control. * indicates statistically significant differences ($p < 0.05$). f.w. – fresh weight

The isolates selected to integrate the consortium were further characterized combining a genetical analysis and morphological traits. The phylogenetic analysis supported the assignment of the three filamentous cyanobacteria (isolates 1.9A, 2.2B and 2.1A) to the genera *Trichocoleus, Nodosilinea,* and *Microcoleus,* respectively. Cyanobacteria belonging to these genera have been often found in soils/biocrusts (Couradeau et al. 2019; Mehda et al. 2021; Mühlsteinová et al. 2014; Radzi et al. 2019; Roncero-Ramos et al. 2019; Samolov et al. 2020), for e.g. *Trichocoleus* strains are common in desert soils (Mühlsteinová et al. 2014; Zhang et al. 2016), *Microcoleus* is considered one of the cosmopolitan biocrust key taxa (with *M. vaginatus* usually being the dominant member of the biocrust) (Couradeau et al. 2019; Mehda et al. 2021; Roncero-Ramos et al. 2019), and representatives of the genus *Nodosilinea* have been also identified in soil/biocrusts, namely in desertic and Antarctica regions (Mehda et al. 2021; Perkerson et al. 2011; Radzi et al. 2019). The filamentous isolate 2.1B belongs to the green algal genus *Klebsormidium* that has also been found worldwide in various habitats and is a key taxa in biocrusts (Borchhardt and Gründling-Pfaff 2020; Mikhailyuk et al. 2015; Ryšánek et al. 2016; Samolov et al. 2019, 2020). The two heterocystous nitrogen-fixing cyanobacteria (isolates 1.11 and 2.7) belong to the *Nostoc* genus, but most probably to two different species. Many studies have shown that *Nostoc* strains and other heterocystous cyanobacteria are widely found in biocrusts (Mehda et al. 2021; Roncero-Ramos et al. 2019; Zhang et al. 2016). These cyanobacteria fix atmospheric nitrogen, an important process that allow them to supply significant amounts of combined nitrogen to the ecosystem and their use in agriculture is well documented (Chittora et al. 2020; Iniesta-Pallarés

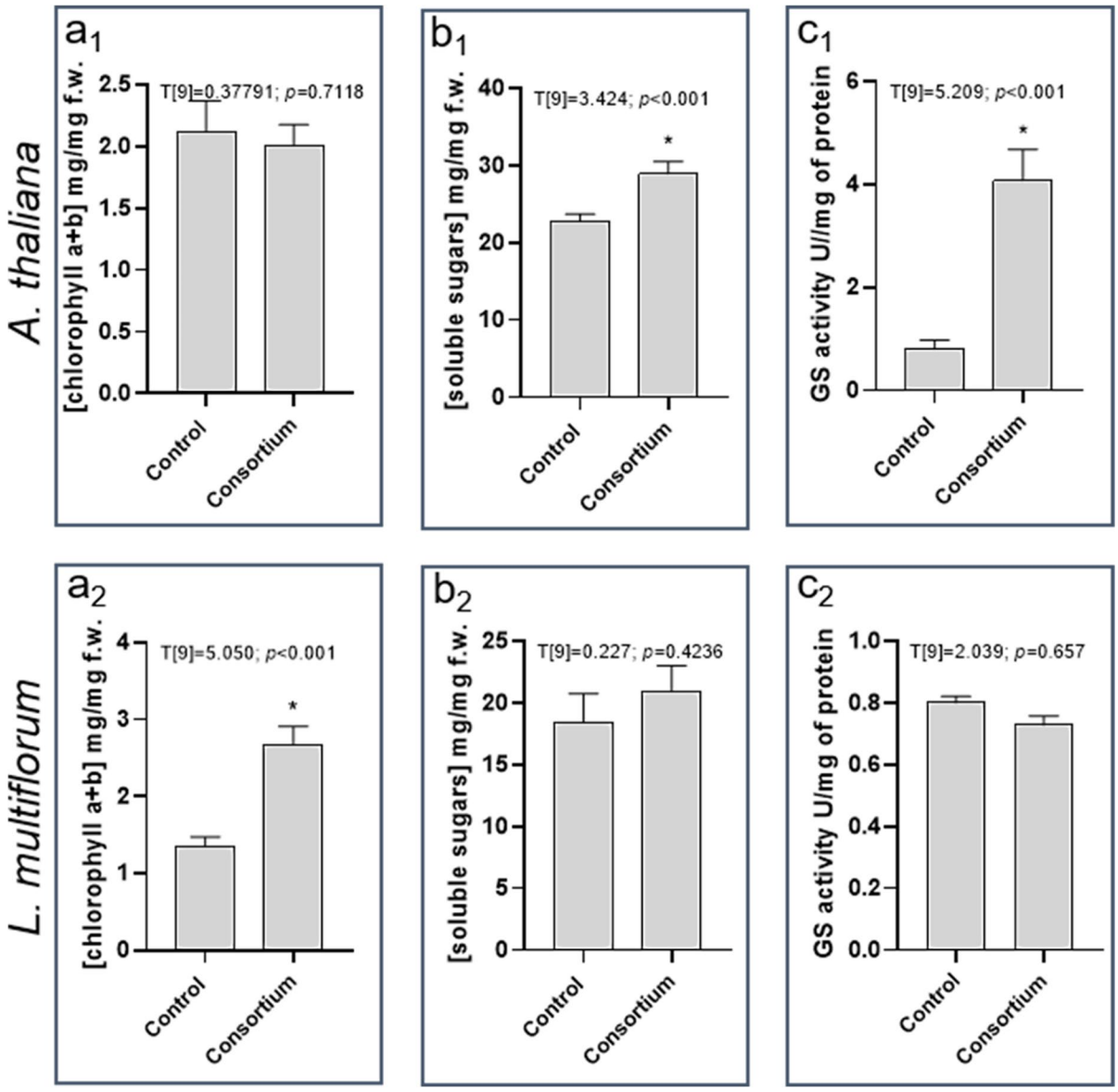


Fig. 12 Effect of the microorganisms' consortium on *Arabidopsis thaliana* and *Lolium multiflorum* productivity. *A. thaliana* ($\mathbf{a_1}$-$\mathbf{c_1}$) and *L. multiflorum* ($\mathbf{a_2}$-$\mathbf{c_2}$) plants were grown in hydroponic cultures with Hoagland medium (Control) or Hoagland medium supplemented with the microorganisms' consortium (Consortium) (for details see Table 2). The total chlorophyll ($\mathbf{a_1}$,$\mathbf{a_2}$) and soluble sugars ($\mathbf{b_1}$,$\mathbf{b_2}$) content and enzymatic activity of glutamine synthetase—GS ($\mathbf{c_1}$,$\mathbf{c_2}$) were evaluated. The results are expressed as means ± SE. Unpaired Student's t-tests, with Welch's correction, were performed, comparing treated plants to the control. * indicates statistically significant differences ($p < 0.05$). f.w. – fresh weight

et al. 2021; Kollmen and Strieth 2022; Lee and Ryu 2021). Although our phylogenetic analysis may suggest that some of the organisms characterized here could be new species, further studies are required to validate this claim.

Overall, growing evidence supports that the inoculation of soils with filamentous photosynthetic microorganisms enhance biocrusts formation (Abinandan et al. 2019), as demonstrated with inoculation with *M. vaginatus* in desert soils (Wang et al. 2009), *K.*

subtile in sandy soils (Lichner et al. 2013), and some of our isolates (in particular the *Trichocoleus* sp.) in microcosm experiments (Gonçalves et al. 2022). They also seem to have a pivotal role on the improvement of soil conditions, due to the intertwined of the filaments and the extracellular matrix (mostly composed of exopolysaccharides) that aggregate soil particles and enhance water retention (Abinandan et al. 2019; Chamizo et al. 2018). This extracellular matrix is particularly evident for one of our *Nostoc* strains (isolate 1.11), that forms compact gelatinous colonies.

Here, the co-cultivation of plants with exudates from *Trichocoleus, Nodosilinea, Klebsormidium* (filamentous) and the *Nostoc* sps. (diazotrophic) revealed that the biostimulation goes beyond their direct effect in soil. In fact, it has been increasingly reported that cyanobacteria and microalgae produce numerous compounds with plant growth-promoting effects, and several *Nostoc* and filamentous cyanobacterial strains have been successfully used to boost crop growth, like corn wheat, rice or bean, most probably due to the production of phytohormones (for recent reviews see Alvarez et al. 2021; Kapoore et al. 2021; Mutale-Joan et al. 2023; Santini et al. 2021). Here we demonstrated that two of our isolates—*Trichocoleus* sp. (1.9A) and *Nostoc* sp. (1.11)—secrete IAA which may contribute to some of the beneficial effects observed. In addition, we could also show that in limited iron conditions the two heterocystous cyanobacteria (isolates 1.11 and 2.7) are able to produce siderophores. The production of these low-weight, high-affinity iron chelating molecules can represent an advantage by promoting iron sequestration not only for the microorganisms but also in the rhizosphere making it available to the plants (Lurthy et al. 2021).

Consortia of cyanobacteria and microalgae with different potential traits and synergistic effects on soil properties and plant growth are currently seen as a valuable strategy for increasing the performance of biofertilizers (Kapoore et al. 2021; Renuka et al. 2018). Indeed, the consortium established in this work showed biostimulant potential in hydroponic cultures of the dicotyledonous *A. thaliana* and the monocotyledonous *L. multiflorum*, increasing several biometric parameters and enhancing physiological productivity related parameters (such as chlorophyll, sugar content and the activity of glutamine synthetase), suggesting an improvement of plant vigour, higher photosynthetic activity and nitrogen use efficiency. Although the mechanisms of action are still largely unknown, it was also recently reported that tomato plants treated with cyanobacteria/microalgae extracts showed increased chlorophyll levels, photosynthetic activity and nitrogen uptake (Mutale-joan et al. 2020, 2021; Rachidi et al. 2020). The increase in the activity of glutamine synthetase reported here, is consistent with the increase in N uptake reported by Mutale-joan et al. (2020), and the rise of other nitrogen metabolism enzymes (Rachidi et al. 2020). The higher chlorophyll content and photosynthetic activity can be a consequence of the enhanced nitrogen metabolism or due to a protective effect that impacts chlorophyll degradation (Mutale-joan et al. 2020; Santini et al. 2021). Here, the evaluation of stress related parameters revealed that the presence of the microorganisms did not lead to a stressful situation as the cellular membrane oxidative damage measured by the level of lipid peroxidation (Alché 2019) and H_2O_2 (Smirnoff and Arnaud 2019) remained unchanged in both plant species. However, an increase of the non-enzymatic antioxidant defensive molecule proline (Ghosh et al. 2022; Szabados and Savouré 2010), and a significant decrease of the enzymatic anti-oxidant system, namely catalase and aspartate peroxidase (Gill and Tuteja 2010) were observed. The increase in proline may grant higher protection to the plants, decreasing the need for the enzymatic system. A strong correlation between proline levels and electric conductivity of the nutritive solution was shown by other authors (e.g. Pirlak and Eşitken 2004) but with an increment of 3 mS cm^{-1}, however here the increase in proline cannot be exclusively attributed to an increase in the osmolarity of the hydroponic solution induced by the presence of the microorganisms, since only a minor difference was detected. Increase in proline content was previously detected in lettuce and tomato plants co-cultivated with cyanobacteria or treated with microalgae-cyanobacteria extracts, most probably contributing to the observed mitigation of the effects of salinity stress (Brito et al. 2022; Mutale-joan et al. 2021). In addition, proline may act as a signal molecule controlling plant development and promoting growth and photosynthetic activity under non-stressful conditions (Guan et al. 2019; Mattioli et al. 2009; Wang et al. 2014; Ambreen et al. 2021). Therefore, the increased levels of proline observed in this work may play a role in the biostimulant effect triggered by the microorganisms.

In summary, the present study contributes to the knowledge on the biodiversity of native cyanobacteria and microalgae from Portuguese soils and shows their potential as biostimulants. Furthermore, constitutes a step forward towards understanding the effect of these microorganisms/compounds released by these microorganisms on higher plants and shed light on the role of proline in these interactions. Considering the results obtained, this virtually untapped resource of native soil microorganisms can, in the future, be used to boost crop production and/or the recovery of damaged soils.

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Author contributions PT, PM, AB contributed to the study conception and design. Experimental work was mainly performed by JR, MR, JP, TN, MB. Data analysis were performed by JR, AB, JV, CPV, PM, PT. Writing was conducted by AB, PM and PT with contributions from JR, JV and CPV. All authors read and approved the final manuscript.

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Data availability Data that support the findings of this study have been deposited in GenBank with the accession codes: OK161231, OK161232, OK161253, OK161269, OK161270 and OQ320396. The authors declare that all the other data supporting the findings of this study are available within the article and its supplementary information file.

Code availability Not applicable.

Declarations

Ethics approval Not applicable.

Competing interests The authors have no relevant financial or non-financial conflicts of interest to declare.

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