# A new cyanobacterial species with a protective effect on lettuce grown under salinity stress: Envisaging sustainable agriculture practices

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## Abstract

In this work, a new terrestrial cyanobacterial species, *Oculatella lusitanica* LEGE 161147, was isolated and characterized using a polyphasic approach. Morphologically, *O. lusitanica* shares characteristics with different *Oculatella* species (mainly with *O. crustae-formantes*), lacking distinctive features. However, the phylogeny based on the 16S rRNA gene sequence and the 16S-23S ITS secondary structures support the establishment of this isolate as a new species. *O. lusitanica* is placed within a clade mainly composed by other *Oculatella* terrestrial strains; however, it forms a separate lineage. In addition, our species differs from the other *Oculatella* described so far by lacking the V2 helix within the ITS region. Since cyanobacteria are known to release compounds that promote plant growth and/or increase their tolerance to stresses, the effect of this newly described cyanobacterial species on *Lactuca sativa* (lettuce) plants development and salinity stress resistance was evaluated. Our results showed that, although the cyanobacterium had no impact on plant growth under the conditions tested, it was able to mitigate the deleterious salinity stress effects on plant size, root and aerial part fresh weight, by eliciting the non-enzymatic antioxidant response system (proline, $H_2O_2$ and reduced glutathione). In addition, the microorganism was able to induce a priming effect on lettuce plants by stimulating defensive mechanisms under non-stress conditions, and enhances the activity of nitrogen metabolism-related enzymes glutamate dehydrogenase, glutamine synthetase and nitrate reductase. These results indicate that this native terrestrial cyanobacterial species could be employed as a tool in sustainable agricultural practices.

**Keywords** Biostimulants · Cyanobacteria · Lettuce · *Oculatella lusitanica* · Phylogeny · Salinity stress

## Introduction

Due to their morphological and metabolic versatility, cyanobacteria are able to colonize and inhabit a variety of habitats ranging from aquatic to terrestrial, including extreme ones (Whitton 2012). Despite playing many essential roles in terrestrial environments, the knowledge concerning these cyanobacterial strains and their biodiversity is far from the information gathered about their aquatic counterparts. Being photoautotrophic organisms, they are pioneers on the establishment of biological soil crusts (biocrusts) as well as on the colonization of various rocks and minerals, including both

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natural and anthropogenic substrates such as sandstone, granite, limestone and clay, leading to the formation of complex microbial mats (Hauer 2007; Hauer et al. 2015). Therefore, they can be considered "ecosystem engineers" due to their ability to modify, maintain and create habitats for other organisms (Jones et al. 1994). Recent studies also demonstrated that cyanobacteria contribute to the improvement of soil properties, water retention and soil fertility (N and C fixation), as well as soil structure and stability (filamentous forms and exopolysaccharides) (Chamizo et al. 2012, 2016, 2018; Rossi and De Philippis 2015; Roncero-Ramos et al. 2019). Furthermore, it has been shown that cyanobacteria have the ability to secrete a number of substances that promote plant growth and development, such as phytohormones and vitamins (Singh 2014; Grzesik et al. 2017), and/or compounds that can mitigate the adverse effects of biotic and abiotic stresses (Poveda 2021). Recently, cyanobacteria have also been successfully utilized for the colonization and restoration of degraded and burned soils (Rossi et al. 2017; Chamizo et al. 2020; GR et al. 2021).

Among the cyanobacterial genera isolated from terrestrial habitats, one that has been frequently reported is *Oculatella* Zammit, Billi *et* Albertano, a member of the family Oculatellaceae (Synechococcales) (Mai et al. 2018). This genus was recently separated from *Leptolyngbya* Anagnostidis & Komárek, a polyphyletic genus in which the species were mainly established using morphological traits (Zammit et al. 2012). The filamentous non-heterocystous *Oculatella* genus can be morphologically distinguished from all the other filamentous cyanobacteria, including other *Leptolyngbya*-like taxa, by the presence of a typical rhodopsin-like reddish inclusion at the tip of the mature apical cells (Albertano et al. 2000). This photoreceptive structure is most probably associated with the positive phototaxis displayed by *Oculatella* (Albertano et al. 2000; Zammit et al. 2012).

The type species, *O. subterranea* Zammit, Billi *et* Albertano, is characterized by reddish trichomes and a hypogean habitat (Zammit et al. 2012). Subsequently, twelve additional species have been described originating from different habitats, but mostly from terrestrial ones (Zammit et al. 2012; Osorio-Santos et al. 2014; Vinogradova et al. 2017; Becerra-Absalón et al. 2020; Jung et al. 2020).

In this study a cyanobacterial strain was isolated from a clay pot (Central Portugal) and characterized at morphological (light and electron microscopy) and molecular level (16S and ITS rRNA, phycobiliprotein content) leading to the description of a new species: *Oculatella lusitanica* sp. nov. Since cyanobacteria are known to produce compounds that enhance plant growth and/or tolerance to abiotic stresses, the potential of this newly described cyanobacterial species as biostimulant for lettuce (*Lactuca sativa* L.) plants growth and mitigation of salinity stress was evaluated.

## Material and methods

### Sampling, isolation and culture conditions of the cyanobacterium

The biological sample was collected by scraping from the inside of a clay pot at Quinta do Castro, Anadia, Central Portugal in June 2016 (40°26'37.15"N; 8°26'33.03"W) (see Fig. 1). The isolation procedure included liquid culture enrichment in BG11 medium containing 1.5 g L$^{-1}$ NaNO$_3$ (Stanier et al. 1971) and when visible growth was observed, an aliquot was streaked onto solid BG11 plates (1.2% w/v agar, Lebkem). The isolation took advantage of this cyanobacterium gliding motility and phototactic ability. Small amounts of biomass were re-streaked in fresh BG11 plates (1% w/v agarose instead of agar) and transferred into liquid medium. To prevent the growth of eukaryotic microorganisms, both types of media were supplemented with cycloheximide at a final concentration of 100 μg mL$^{-1}$ (Rippka 1988). During culture growth, agarose plates were partially covered with aluminum foil and placed under a 12 h light/12 h dark cycle and lateral illumination (20 μmol photons m$^{-2}$ s$^{-1}$), at 19 °C. Before and after transfers, liquid and solid cultures were visually inspected under a microscope to ascertain the success of the isolation. These processes were repeated until isolation was achieved. The isolated unicyanobacterial strain—*Oculatella lusitanica* LEGE 161147—was maintained at 25 °C under a 16 h light (15–25 μmol photons m$^{-2}$ s$^{-1}$)/8 h dark regimen with orbital shaking (80 rpm) and deposited at LEGE Culture Collection (CIIMAR, Matosinhos, Portugal). Voucher specimen was deposited at the Herbarium for Nonvascular Cryptogams (CBFS) at the Department of Botany, Faculty of Science, University of South Bohemia, Czech Republic, with the accession number CBFS A-125-1 and at the Herbarium of University of Porto, Porto, Portugal, with the accession number PO-T4782.

### Morphological and ultrastructural characterization

Cells were observed using an Olympus CX31 light microscope (Olympus, Japan) and micrographs were acquired with an Olympus DP25 camera and the Cell^B image software (Olympus, Japan).

For transmission electron microscopy (TEM) studies, cells were collected, centrifuged at 2500x*g* for 10 min. and processed as previously reported (Santos et al. 2021). Ultrathin sections were examined using a JEM-1400Plus (JEOL, USA) electron microscope operating at 80 kV.

### Molecular characterization

Genomic DNA was extracted using the Maxwell ®16 System according to the instructions of the manufacturer. PCR amplifications of the regions encoding part of 16S rRNA,

**Fig. 1** *Oculatella lusitanica* LEGE 161147 was isolated from the inside of clay pot (**a**) at the Central Region of Portugal (**a** insert). The initial mat contained also green algae and at least another cyanobacterial strain (**b** and respective insert). Typical aggregation of the filaments that develops in liquid medium with orbital shaking (**c** insert), and optical micrographs of the unicyanobacterial culture (**c** to **d**) showing the flexuous filaments (**c**), mature filaments with the characteristic reddish-orange eyespot in the apex of the apical cells (**d**) and the breakage of a trichome within the sheath (**d** insert). TEM longitudinal and transversal sections of the filaments (**e** to **h**) in which is possible to observe the rounded or rounded conical shape of the apical cells (**e**, **f**), the parietal arrangement of the thylakoids (th), the sheath (sh) and the cell envelope (ce). Scale bars: b, c = 50 µm; d = 20 µm; e, f = 1 µm; g = 0.5 µm; h = 0.2 µm.
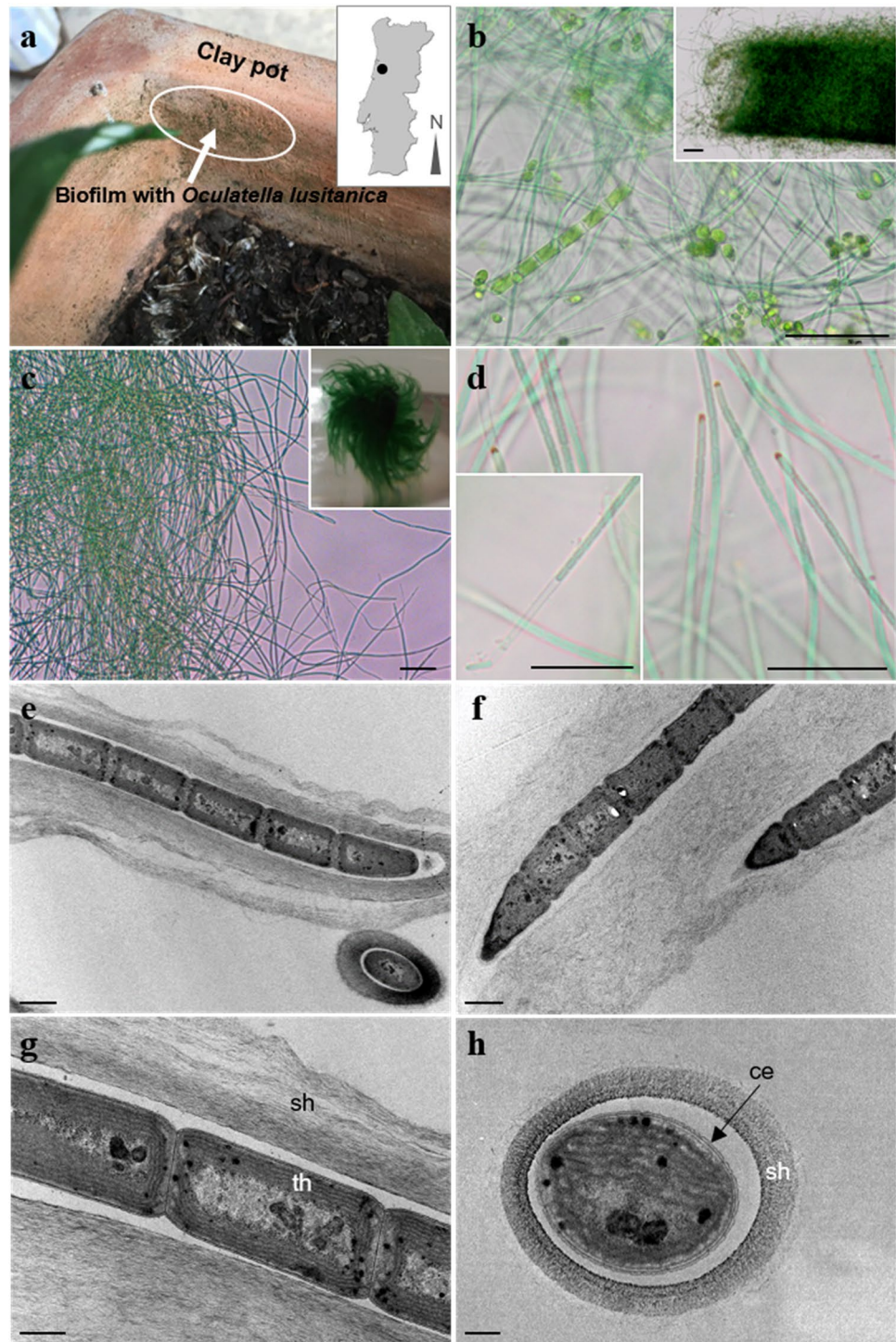


the internal transcribed spacer (ITS) and most of the 23S rRNA gene were performed using the oligonucleotide primers listed in Table S1. PCR reactions were carried out using a thermal cycler (MyCycler™, Bio-Rad Laboratories, USA) following procedures previously described (Tamagnini et al. 1997). The PCR profiles included an initial denaturation at 94 °C for 5 min, followed by 35 or 30 cycles (for ITS) at 94 °C for 1 min, 52 °C or 60 °C (for ITS) for 1 min, 72 °C for 1 min and a final extension at 72 °C for 7 min. PCR products were separated by agarose gel electrophoresis (Sambrook and Russell 2001) and DNA fragments were isolated from gels using the NZYGelpure Kit (NZYTech, Lisbon, Portugal), according to the manufacturer's instructions. Purified products were cloned into pGEM®-T Easy vector (Promega,

USA) and transformed into *Escherichia coli* DH5α competent cells following the manufacturer's instructions, and the methodology previously described (Ramos et al. 2010). Some purified PCR products were directly sequenced at STAB Vida (Lisbon, Portugal). Sequence data were deposited in the GenBank database under the accession number MN061918.

## Phylogenetic analysis

The 16S rRNA gene sequence from *Oculatella lusitanica* LEGE 161147 was searched against the NCBI BLASTn database (January 2021), in order to find the most similar sequences, and the ones above 95% similarity were retrieved. In addition, in order to have a reliable and robust backbone representation of the *Oculatella* genus, sequences from all described species so far, including reference strains, were also included in this analysis. In total, 73 sequences were used, including *Gloeobacter violaceus* PCC 7421 (BA000045), and two *Leptolyngbya boryana* sequences (AF132793 and NZKB731324) as outgroup. Sequences were aligned using Clustal Omega (Sievers et al. 2011) and phylogenetic relationships inferred using maximum likelihood (ML) [Fasttree; (Price et al. 2009, 2010)], using 500 bootstrap replicates. The model used in the ML analyses was the general time reversible model with a proportion of invariant sites and a gamma distribution (GTR + I + G), since this is the selected model when using the Akaike information criterion (AIC), as implemented in jModeltest2 (Guindon and Gascuel 2003; Darriba et al. 2012). *Gloeobacter violaceus* PCC 7421 (BA000045) was used to root the tree using MEGA7 (Kumar et al. 2016). A total of 877 ungapped and non-ambiguous positions are used in phylogenetic analyses. DnaSP (Rozas et al. 2017) was used to estimate polymorphism and divergence values. All software applications here used are available as Docker images at the pegi3S Bioinformatics Docker Images Project (https://pegi3s.github.io/dockerfiles/).

The secondary structures of the different domains of the 16S-23S ITS region, including D1-D1', Box-B and V3 helices, were folded individually using CLC Genomics Workbench software 21 (CLC Bio-Qiagen, Aarhus, Denmark). Default parameter settings were used in this analysis.

## Extraction of phycobiliproteins

For phycobiliproteins extraction, cells were collected, centrifuged at 2500x*g* for 10 min. and processed as previously described (Brito et al. 2017). Absorbance spectrum was recorded using an UV-2401PC spectrophotometer (Shimadzu, Japan) in the range 350–750 nm.

## Plant material and growth conditions

Seeds of *Lactuca sativa* L. cv Marvel of Four Seasons (Vilmorin, France) were superficially sterilized with 10% (v/v) commercial bleach, washed, soaked in distilled water and germinated in pots with vermiculite/perlite (2:1) substrate (Control), or with the substrate supplemented at the top with the perlite in which *O. lusitanica* was previously grown for one month (Oculatella). The plants were irrigated twice a week with Hoagland solution (Sigma, USA), pH 5.70 (Control and Oculatella) or with the same solution supplemented with NaCl (100 mM) (NaCl and Oculatella NaCl). The germination and the plants growth occurred under controlled conditions [16 h light (100 µmol photons m$^{-2}$ s$^{-1}$)/8 h dark at 23 ± 2 °C]. After four weeks, the plant biometric parameters (plant size, number of leaves, and root and aerial part fresh weight) were assessed. For the *O. lusitanica* growth, the perlite was washed, sterilized and soaked in BG11 medium; the growth conditions for the microorganism were the ones mentioned above.

## Quantification of proline, glutathione, H$_2$O$_2$ and lipid peroxidation

For proline and glutathione (GSH) quantification, plant material (200 mg) was homogenized using 3% (w/v) sulfosalicylic acid and centrifuged at 500x*g* for 10 min. The proline was detected in the supernatant with acidic ninhydrin, after extraction of the formed chromophore with toluene, as previously described (Bates et al. 1973), and the GSH was detected using Ellman's Reagent [DTNB: 5,5-dithiobis-(2-nitrobenzoic acid)] according to Rahman et al. (2006), using standard curves for quantification.

For H$_2$O$_2$ and lipid peroxidation quantifications, plant material (200 mg) was homogenized in 0.1 % (w/v) trichloroacetic acid (TCA) and centrifuged at 10 000x*g* for 5 min. The content in H$_2$O$_2$ was determined spectrophotometrically according to Alexieva et al. (2001), by reaction with potassium iodide ($\varepsilon$390 nm = 0.28 µM$^{-1}$ cm$^{-1}$). Lipid peroxidation was estimated according to Heath and Packer (1968), by determining the malondialdehyde (MDA) levels spectrophotometrically after the reaction with 0.5% (w/v) thiobarbituric acid (TBA) in 20% (w/v) TCA ($\varepsilon$532 nm of 155 mM$^{-1}$ cm$^{-1}$).

## Enzyme extraction and quantification of activity

The procedure for the quantification of the enzymes activities was initiated by extracting the soluble proteins with appropriate extraction buffer, on ice, followed by centrifugation at 15 000x*g* at 4 °C for 15 min. The soluble protein concentration was measured by the Bradford Dye Reagent assay (Alfa Aesar), using BSA as a standard. For the antioxidant enzymes (AOX), superoxide dismutase (SOD, E.C. 1.15.1.1), catalase

(CAT, E.C. 1.11.1.6) and ascorbate peroxidase (APX. EC 1.11.1.11), plant material was homogenized in extraction buffer containing 100 mM potassium phosphate buffer, pH 7.3, 1 mM ethylenediaminetetraacetic acid, 8% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, 5 mM ascorbic acid and 2% (w/v) polyvinylpolypyrrolidone. SOD activity was spectrophotometrically quantified, based on the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm (Donahue et al. 1997). CAT activity was estimated by measuring the catalase-mediated degradation of $H_2O_2$ ($\varepsilon 2400nm = 39.4$ mM$^{-1}$ cm$^{-1}$), as described by Aebi (1984). APX activity was determined by monitoring ascorbic acid oxidation ($\varepsilon 290nm$ of 0.49 M$^{-1}$ cm$^{-1}$), following Murshed et al. (2008) protocol. For the glutamate dehydrogenase (GDH, EC 1.4.1.2), glutamine synthetase (GS, EC 6.3.1.2) and nitrate reductase (NR, EC 1.6.6.1-3) activity quantification, the following extraction buffers were used for proteins extraction: GDH extraction buffer (50 mM HEPES, pH 7.5, 10% (v/v) glycerol, 0.05% (w/v) BSA, 1 mM phenylmethylsulfonyl fluoride, 0.05% (v/v) Triton X-100, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA and 10 mM dithiothreitol), GS extraction buffer (10 mM Tris-HCl pH 7.5, 5 mM Na-glutamate, 10 mM $MgSO_4$, 1 mM dithiothreitol, 10% (v/v) glycerol, 1.5% (w/v) polyvinylpolypyrrolidone, 1 mM phenylmethylsulfonyl fluoride, 0.05% (v/v) Triton X-100) and NR extraction buffer (50 mM HEPES-KOH, pH 7.8, 1 mM PMSF, 10 mM $MgCl_2$). GDH activity was determined, in the aminating direction, by monitoring the oxidation of NADH spectrophotometrically at 340 nm and using a NADH standard curve, as described by Sarasketa et al. (2014). GS activity was determined by quantification of the α-glutamyl hydroxamate, at 530 nm, produced by the transferase reaction as previously described by Cullimore and Sims (1980). NR activity was measured following the consumption of NADH ($\varepsilon 340$ nm = 6, 22 mM$^{-1}$ cm$^{-1}$), as described by Kaiser and Brendle-Behnisch (1991).

## Statistical analysis

Three independent experiments, using 10–12 plants, were performed. For the biochemical quantification, each experiment was treated as a pool, with at least 3 independent technical replicates, with the results expressed as mean ± standard deviation (SD). 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 ANOVAs using a Tukey's multiple comparison test ($p < 0.05$) for analysis of 3 or more datasets, both performed using the GraphPad Prism 7 software (GraphPad Software Inc., USA).

# Results

The present study reports on the isolation and description of a filamentous cyanobacterial strain from a mat present inside of a clay pot, Central Portugal (Fig. 1a, b). The detailed characterization, combining morphological and molecular data, leads to the description of a new *Oculatella* species: *Oculatella lusitanica*. Since cyanobacteria are known to release biologically active substances promoting plant growth and/or increase their tolerance to stresses, we evaluated the effects of this native strain on *L. sativa* (lettuce) plants development and salinity stress resistance.

## Taxonomic description

*Oculatella lusitanica* Brito, Tamagnini, Melo *et* Kaštovský, sp. nov. (Fig. 1)

Thallus flat, thin mat, blue-green, filaments flexuous, rarely with more than one trichome in common mucilage, with sheath 1.0–1.5 µm wide. Sheath hyaline, non-lamellate, colorless, in older filament rarely widened with wavy edges. Trichomes motile constricted at the crosswalls, without granulation at the crosswalls or in cytoplasma, parietal position of thylakoid clearly visible in electron microscopy micrographs (Fig. 1c–h). False branching lacking, necridic cells not produced. Cells consistently longer than wide, 0.9–1.1 (1.2) µm wide and (1.2) 1.5–1.8 (2.2) µm long. Apical cells rounded or rounded conical, generally longer than vegetative cells (1.9–5.6 µm) (Fig. 1d–f), with a reddish-orange spot in the apex (Fig. 1d).

Contains phycocyanin (PC) but not phycoerythrin (PE) (Fig. S1).

**Habitat:** Aerophytic, isolated from a clay pot, Portugal.

**Type locality:** Clay pot with soil, garden, Anadia, Portugal. Collected by Paula Melo, June 2016.

**Holotype here designated:** Holotype CBFS A-125-1, Herbarium for Nonvascular Cryptogams at the Department of Botany, Faculty of Science, University of South Bohemia, Czech Republic. Isotype PO-T4782, Herbarium of University of Porto, Porto, Portugal.

**Reference strain:** *Oculatella lusitanica* LEGE 161147.

**Etymology:** Lusitanica—from Portugal.

**Morphological comparison with other species:** *Oculatella lusitanica* does not have distinct morphological characteristics, sharing morphological features with the different *Oculatella* species. It is blue-green as the majority of the species described, differing in color from the type species, *Oculatella subterranea* Zammit, Billi & Albertano, which is reddish. While *O. subterranea* contains PC and PE, *O. lusitanica* contains only PC. The width of *O. lusitanica* filaments (1.0–1.5 µm) is similar to that reported for *O. crustae-formantes* (0.8–1.6 µm), the species with the thinner

filaments described within the genus, and to *O. cataractarum* (1.3–1.7 μm) and *O. kauaiensis* (1.2–1.7 μm). The trichome width is also in the same range of the three mentioned species. Similarly to *O. crustae-formantes* (1.2–2.9 μm), *O. lusitanica* has shorter cell length (1.5–3.0 μm) compared to other species. In addition, it does not have granules at crosswalls as reported for some strains (*O. subterranea*, *O. kauaiensis*, *O. coburnii*, *O. leona* and *O. dilatativagina*). A morphological comparison between *O. lusitanica* and all known *Oculatella* species is depicted in Table 1, and the determination key for *Oculatella* genus leading to the identification of *Oculatella lusitanica* is shown in Fig. S2.

## ITS secondary structures

The 16S-23S ITS region of *O. lusitanica* displays 537 nucleotides and both tRNA$^{Ile}$ and tRNA$^{Ala}$ with 74 and 73 nucleotides long, respectively, were identified. The secondary structures of the main helices were determined (Fig. 2) and compared to the correspondent ones from all *Oculatella* species. The V2 helix was not identified in *O. lusitanica*; thus, our species differs from the others by lacking this particular region. In contrast, the D1-D1', Box-B and V3 helices in *O. lusitanica* are similar to the ones described for the other species. The D1-D1' helix of *O. lusitanica* is 64 nucleotides long, with a bilateral bulge at nucleotides 16-17/41-42 and the subterminal bilateral bulge at nucleotides 22-24/34-36, a structure similar to those reported for *O. subterranea* and *O. cataractarum.* The Box-B structure is quite similar in all *Oculatella* spp., being generally 34 nucleotides long with 4–7 nucleotides in the terminal loop (6 nucleotides for *O. lusitanica*). The V3 helix of *O. lusitanica* is 52 nucleotides long, with a unilateral bulge on the 5' side of the helix at
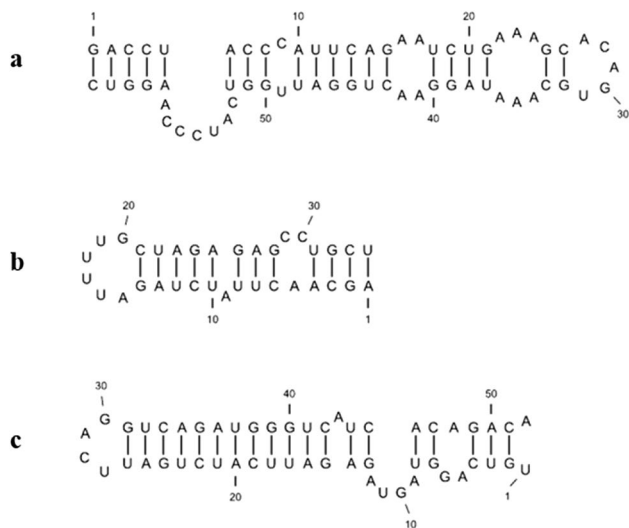


**Fig. 2** Predicted secondary structures of helices of the 16S-23S ITS region of *Oculatella lusitanica* LEGE 161147. **a**—D1-D1'helix; **b**—Box-B helix; **c**—V3 helix.

nucleotides 9–12, a structure similar to those reported for *O. hafneriensis*, *O. mojaviensis*, *O. coburnii*, *O. ucrainica, O. kazantipica, O. dilatativagina* and *O. leona*.

## Phylogenetic analysis

The phylogenetic analysis, based on 16S rRNA gene sequences, showed that *O. lusitanica* falls within the *Oculatella* clade (Fig. 3), supporting the morphological assignment. *O. lusitanica* is placed within a large cluster comprising known terrestrial species *O. atacamensis*, *O. leona*, *O. mojaviensis*, *O. ucrainica* and *O. coburnii*, supported by a strong bootstrap value. However, *O. lusitanica* forms a separated branch together with *Oculatella* sp. LLi18 (from Costa Rica). In fact, the species status of *O. lusitanica* is supported by three observations: (i) levels of polymorphism within recognized species, except for *O. neakameniensis* and *O. kazantipica*, which are always lower than the divergence observed between *O. lusitanica* and *Oculatella* sp. LLi18 (Table S2 and S3); (ii) for *O. ucrainica* and *O. coburnii*, recognized sister species, there is less divergence than that observed for the *O. lusitanica* and *Oculatella* sp. LLi18 (Table S3); (iii) there is no obvious relationship between geography and phylogenetic relationships that could suggest that the observed divergence could be due to differentiation between populations of the same species in different continents.

## Plant growth

Lettuce plants were grown in pots with a mixture of vermiculite and perlite supplemented at the top with perlite containing the cyanobacterium *O. lusitanica*, under standard (Control and Oculatella) or high salinity conditions (NaCl and Oculatella NaCl) (for details see Material and methods). Under standard growth conditions, no differences in size, number of leaves, aerial part and root fresh weights were detected between the plants grown in the presence or absence of the cyanobacterium (Fig. 4). As expected, a marked growth impairment was observed for lettuce plants grown under salinity stress, with a considerable reduction in size and fresh weight of both roots and aerial parts. However, in the presence of the cyanobacterium, the plants under stress displayed a similar growth to the ones grown in standard conditions (Fig. 4).

## Non-enzymatic antioxidant-system

To evaluate the capacity of *O. lusitanica* to stimulate the lettuce defensive responses, we assessed some non-enzymatic stress-related parameters. As expected, the production of proline, $H_2O_2$, reduced glutathione (GSH) and malondialdehyde (MDA) increased in the leaves of the plants grown under salinity stress (Fig. 5). Interestingly, the plants grown

**Table 1** Morphological comparison of *Oculatella lusitanica* sp. nov. with the other *Oculatella* species described so far

| Species | Filament width (μm) | Trichome width (μm) | Cell length (μm) | Constrictions | Crosswalls granules | Sheath | False branching | Necridic cells | Apical cell length (μm) | Color | Habitat |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *O. lusitanica* | 1.0 – 1.5 | 0.9 – 1.1 (1.2) | (1.2) 1.5 – 1.8 (2.2) | clear | absent | common | absent | - | 1.9 – 5.6 | blue-green | clay pot |
| *O. subterranea* | | 1 - 2 | 1.5 - 3 | weak | absent | common | rare | - | | red | hypogea |
| *O. atacamensis* | 1.8 - 4.1 | 1.5 - 2.3 | 1.5 - 7.4 | weak | sometimes | common | rare | - | 2.5 - 9.9 | blue-green | desert soil |
| *O. coburnii* | 1.7 - 2.8 | 1.4 - 1.8 | 1.8 - 4.8 | weak | absent | common | rare | - | 2.4 - 5.4 | blue-green | desert soil |
| *O. mojaviensis* | 2.0 - 2.6 | 1.6 - 2.2 | 1.5 - 5.0 | absent/weak | sometimes | common | rare | + | 2.4 - 6.8 | blue-green | desert soil |
| *O. neaka-meniensis* | 1.2 - 4.1 | 1.2-1.7 | 1.5 - 5.4 | absent/weak | sometimes | common | absent | - | 2.3 - 7.7 | blue-green | soil |
| *O. cataractarum* | 1.3 - 1.7 | 0.8 - 1.3 (1.7) | (1.4) 1.6 - 6.6 (8.7) | absent/weak | frequently | rare | rare | - | 2.1 - 7.7 (12.8) | blue-green | waterfall |
| *O. hafneriensis* | 1.4 - 2.4 | 1.1 - 1.9 | 1.0 - 4.4 | absent/weak | sometimes | common | absent | - | 2.0 - 5.8 | blue-green | lake benthos |
| *O. kauaiensis* | 1.2 - 1.7 | 0.9 - 1.4 | 1.0 - 4.4 | absent/weak | absent | common | absent | - | 1.3 - 7.8 | blue-green | sea cave |
| *O. kazantipica* | (1.3) 1.5 - 5 (7.5) | 1.1 - 1.3 - 1.7 | (2) 2.3 - 4.7 (7.5) | absent/weak | frequently | common | rare | - | (4) 5 - 7 (8.7) | olive-green | conquina beach |
| *O. ucrainica* | (1.5) 2.5 - (3.0) | (1.3) 1.7 - 2.5 (3.0) | (1.3) 1.7 - 3.7 (4.7) | clear | sometimes | common | rare | - | (2.3) 3.3 - 6.7 (7.7) | olive-green | conquina beach, chalk outcrops |
| *O. leona* | 2.9 - 4.4 | 1.5 - 2.7 | 2.4 - 4.4 | weak | absent | common | absent | - | 4.4 - 9 | blue-green | semi-arid soil |
| *O. dilatativa-gina* | 2.2 - 8 | 1 - 1.8 | 2.1 - 4.4 | clear | absent | common | absent | - | 4.4 - 5 | blue-green | semi-arid soil |
| *O. crustae-formantes* | 0.8 - 1.6 | 0.5 - 1.1 | 1.2 - 2.9 | clear | frequently | common | absent | - | 1.5 - 7.7 (9.5) | blue-green | soil crusts |

**Fig. 3** Maximum-likelihood (ML) phylogenetic tree based on partial 16S rRNA gene sequences. The novel *Oculatella* species is highlighted in green and the other 13 species are annotated. *Gloeobacter violaceus* PCC 7421 was used as outgroup. Numbers along branches indicate bootstrap values above 70%, considering 500 pseudoreplicates.
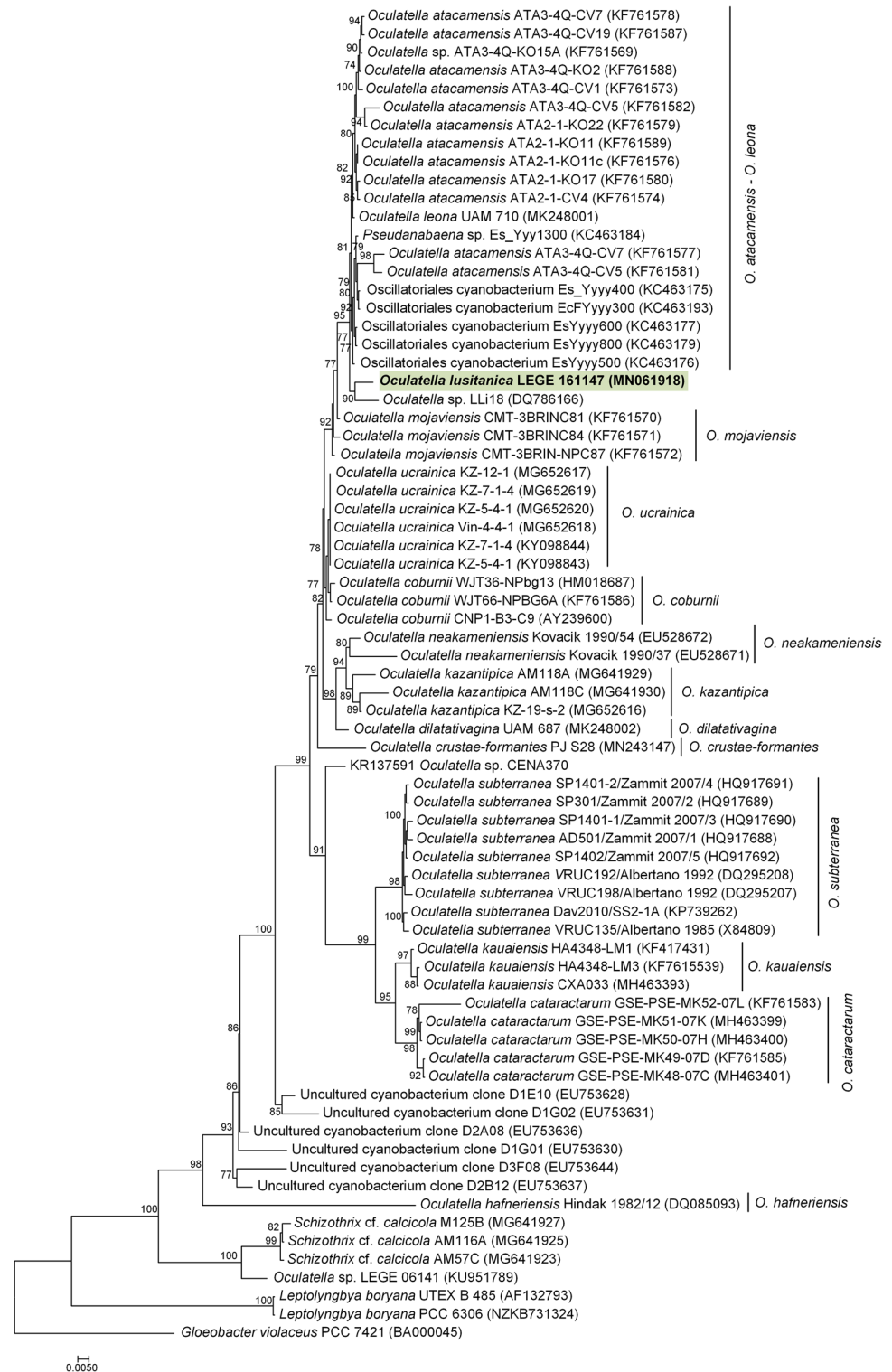
under standard conditions but inoculated with the cyanobacterium, also exhibited higher proline content compared to the control plants (standard conditions/absence of the cyanobacterium). In contrast with this result, under salinity stress the increase in the proline content was lower in the presence of the cyanobacterium (Fig. 5a). Similar results were obtained for the $H_2O_2$ quantifications (Fig. 5b). The presence of the cyanobacterium also increased the GSH content of the leaves, being this content even higher under salinity condition (Fig. 5c). The lipid peroxidation (assessed

**Fig. 4** Effect of *Oculatella lusitanica* LEGE 161147 on *Lactuca sativa* plants growth. The lettuce plants were grown in pots with perlite/vermiculite as substrate in the absence or presence of *O. lusitanica*, and watered with Hoagland solution without [Control and Oculatella (**a**)] or with 100 mM NaCl (NaCl and Oculatella NaCl (**b**)]. Measurements of plant size (**c**), number of leaves (**d**), aerial part fresh weight (**e**) and root fresh weight (**f**). These results are expressed as means +/- SD (n=10). One-way ANOVA tests were performed with Tukey's multiple comparison. Different letters indicate statistically significant differences (p ≤ 0.05).
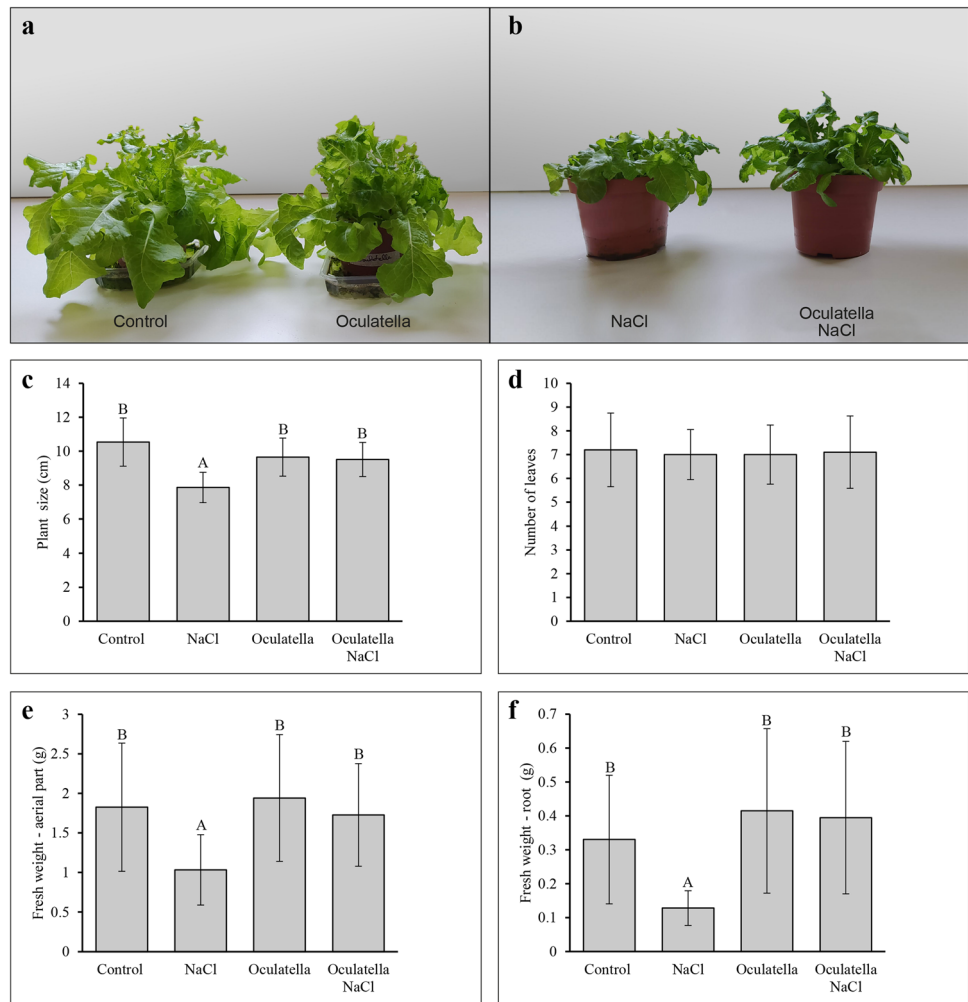
by the MDA quantification), that reflects the integrity of membranes, increases with the salinity stress, being this increase less pronounced in the leaves of the plant grown in the presence of the cyanobacterium (Fig. 5d).

## Enzymatic activities

To evaluate the impact of cyanobacterial inoculation on the antioxidant enzymatic system of plants conferring them protection against salinity stress, the activities of antioxidant enzymes (AOX), catalase (CAT), superoxide dismutase (SOD) and ascorbate peroxidase (APX) were estimated (Fig. 6). While CAT activity increased in plants under salinity stress, both SOD and APX decreased. The inoculation of the cyanobacterium had no significant impact on the activity of all the AOX enzymes, in both standard and salinity conditions (Fig. 6a, b, c).

The activity of glutamate dehydrogenase (GDH), an enzyme of the nitrogen metabolism and considered as a salt stress response enzyme (Tercé-Laforgue et al. 2015), decreased in plants under salinity stress (Fig. 6).

Interestingly, GDH activity increases in the presence of the cyanobacterium under standard conditions (Fig. 6d). As GDH is an important enzyme of the nitrogen metabolism, the activity of other nitrogen metabolism-related enzymes, glutamine synthetase (GS) and nitrate reductase (NR) was evaluated. Under standard conditions, the inoculation with the cyanobacterium led to an increase in the activities of these enzymes (Fig. 7), while under salinity conditions their activities remain unchanged (Fig. S3).

## Discussion

Since the *Oculatella* genus separation from *Leptolyngbya* in 2012 (Zammit et al. 2012), 13 species have been described. *Oculatella* is morphologically characterized by the presence of a reddish eyespot in mature apical cells of the trichrome, and the species initially isolated came from hypogean environments. Subsequently, other species were described, mainly from terrestrial habitats but also from
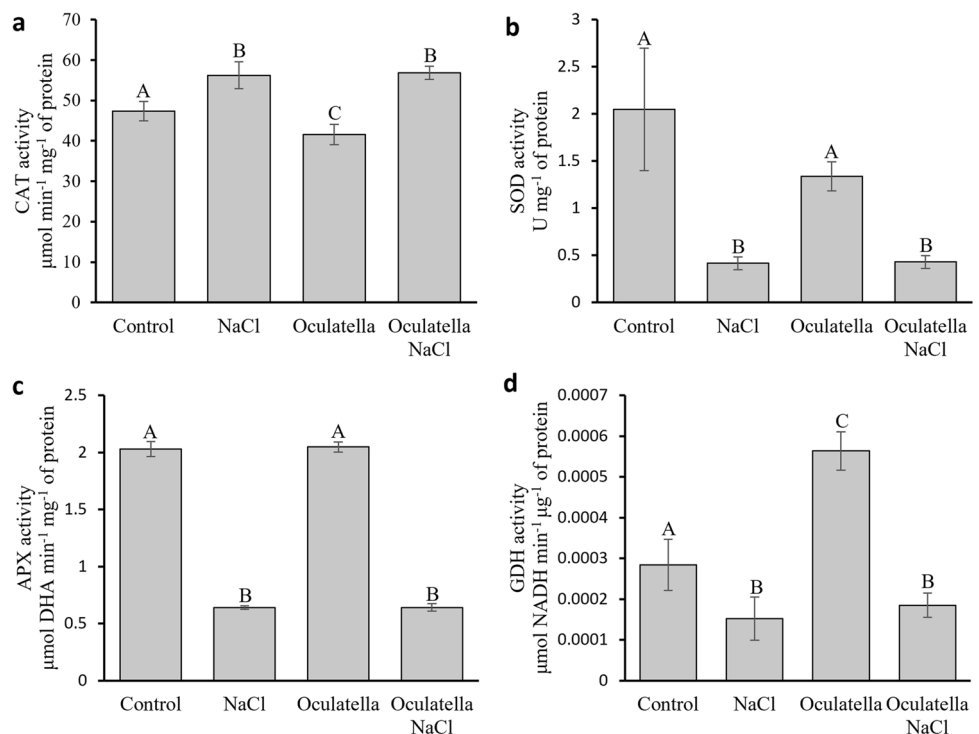
**Fig. 5** Effect of *Oculatella lusitanica* LEGE 161147 on the proline (**a**), H$_2$O$_2$ (**b**), glutathione -GSH (**c**), malondialdehyde—MDA (**d**) content of *Lactuca sativa* plants leaves. The lettuce plants were grown in the absence or presence of *O. lusitanica* and watered with Hoagland solution without (Control and Oculatella, respectively) or with 100 mM NaCl (NaCl and Oculatella NaCl, respectively). The results are expressed as means +/- SD (n=3). One-way ANOVA tests were performed, with Tukey's multiple comparison. Different letters indicate statistically significant differences (p ≤ 0.05).
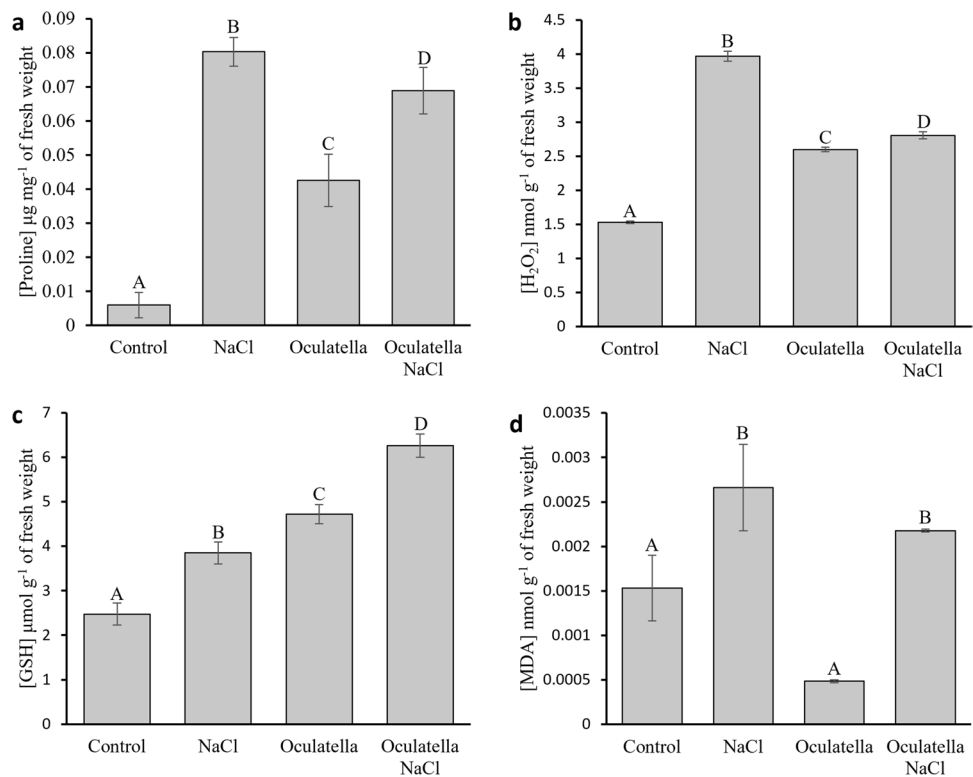


aquatic (e.g., *O. hafneriensis*) environments (Zammit et al. 2012; Osorio-Santos et al. 2014; Vinogradova et al. 2017; Becerra-Absalón et al. 2020; Jung et al. 2020). The new *Oculatella* species described here, *O. lusitanica*, lacks

striking distinctive morphological characteristics sharing most of its traits with *O. crustae-formantes*, namely the width of filaments and trichomes, and the cell length. However, while in *O. crustae-formantes* the granules at

**Fig. 6** Effect of *Oculatella lusitanica* LEGE 161147 on the catalase—CAT (**a**), superoxide dismutase—SOD (**b**), ascorbate peroxidase—APX (**c**) and glutamate dehydrogenase—GDH (**d**) activity in *Lactuca sativa* plant leaves. The lettuce plants were grown in the absence or presence of *O. lusitanica* and watered with Hoagland solution without (Control and Oculatella, respectively) or with 100 mM NaCl (NaCl and Oculatella NaCl, respectively). The results are expressed as means +/- SD (n=3). One-way ANOVA tests were performed, with Tukey's multiple comparison. Different letters indicate statistically significant differences (p ≤ 0.05).
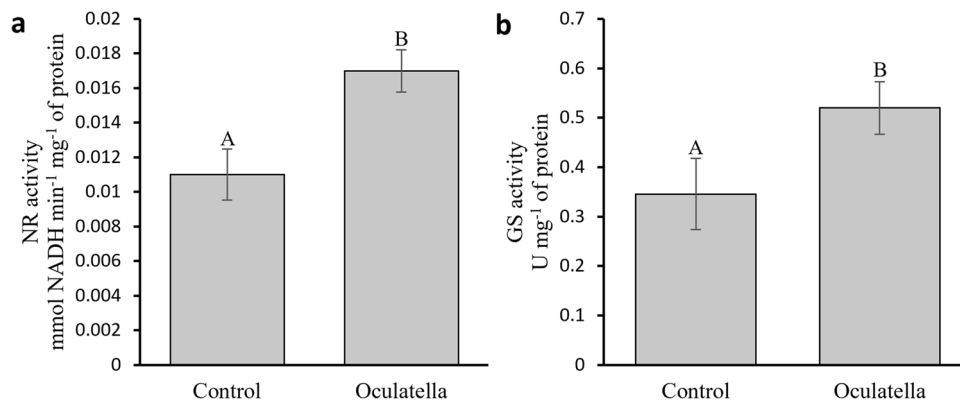
**Fig. 7** Effect of *Oculatella lusitanica* LEGE 161147 on the nitrate reductase—NR (**a**) and glutamine synthetase—GS (**b**) activity in *Lactuca sativa* plant leaves. The lettuce plants were grown in the absence or presence of *O. lusitanica* (Control and Oculatella, respectively) and watered with Hoagland solution. The results are expressed as means +/- SD (n=3). An unpaired t test with Welch's correction assuming two-tailed p values was performed for each analysis. Different letters indicate statistically significant differences ($p \leq 0.05$).

the crosswalls are frequent, in *O. lusitanica* they are absent (Table 1). Nevertheless, at the molecular level (phylogeny of the 16S rRNA gene and ITS secondary structures) it is possible to infer that our strain differs from all the other described *Oculatella* species. This is also valid for other *Oculatella* species (e.g., *O. atacamensis*, *O. coburnii*) that were only recognized using molecular data and, therefore, considered cryptic species (Osorio-Santos et al. 2014). Phylogenetically, *O. lusitanica* forms a separate lineage within the *Oculatella* cluster together with *Oculatella* sp. LLi18, an isolate from a hot volcanic stream at Costa Rica (Fig. 3). The levels of polymorphism found within known species, as well as the divergence observed for *O. lusitanica* and *Oculatella* sp. LLi18 (Table S2 and S3) supported the establishment of this species. It was also possible to observe that *O. lusitanica* is placed within a clade mainly composed by terrestrial strains, namely from biological crusts and arid soils. Since *O. lusitanica* was isolated from a mat exposed to high solar radiation and water stress, it makes sense that it would be closer to the "terrestrial" group of *Oculatella* (*O. atacamensis*, *O. coburnii*, *O. mojaviensis*, *O. leona*, *O. ucrainica, O. neakameniensis, O. kazantipica, O. dilatativagina, O. crustae-formantes*), than to the ones from other habitats (*O. cataractarum, O. hafneriensis, O. kauaiensis, O. subterranea*). The analyses of the secondary structures of ITS further supported the distinctiveness of *O. lusitanica* (Fig. 2). This species lacks the V2 helix, a region identified in all the other *Oculatella* species. Among the ITS helices (D1-D1', Box-B, V2 and V3), V2 is the most variable in structure and number of nucleotides.

Cyanobacteria are increasingly used in agricultural soils mainly due to its potential contribution in plant nutrition (Bocchi and Malgioglio 2010; Singh 2014; Singh et al. 2017; Poveda 2021). In addition, many cyanobacteria release biologically active substances (elicitors molecules) that promote plant growth and/or protect them against biotic and abiotic stresses (Singh et al. 2017; Poveda 2021). Here, we showed that the cyanobacterium *O. lusitanica* attenuated the negative effects induced by high salinity on the growth of lettuce plants, but to date there are no reports on biologically active molecules from *Oculatella* species functioning as plants biostimulants (Santini et al. 2021). Salinity is one of the most harmful environmental factors affecting plant growth and crop productivity (Yang and Guo 2018; Zörb et al. 2019; Soltabayeva et al. 2021). Climate changes and inappropriate modernization of agriculture have increased the incidence of this abiotic stress that is currently affecting a considerable proportion of the world's agricultural land area (Yang and Guo 2018; Zörb et al. 2019). Plants cope with salt stress by activating several defense mechanisms such as the production of enzymatic and non-enzymatic scavengers, and the biosynthesis/accumulation of compatible osmolytes (Yang and Guo 2018; Soltabayeva et al. 2021). To gain a better understanding of the biochemical mechanisms leading to the increased plant tolerance induced by *O. lusitanica*, several physiological parameters were evaluated. The proline content increased sharply in the leaves of lettuce irrigated with a NaCl solution. Proline is a compatible osmolyte with protective functions in osmotic adjustment, detoxification and free-radical scavenging that is produced in plants under adverse environmental conditions as salinity stress. Furthermore, proline acts as signaling molecule, triggering specific gene expression (Szabados and Savouré 2010; Yang and Guo 2018). In contrast, in lettuce plants inoculated with the cyanobacterium a reduced increase in proline production was observed under salinity stress. Similar results were reported for other plant species

exposed to plant growth promoting rhizobacteria (PGPR), algal extracts or mycorrhizal fungal inocula, under drought and salt stress reflecting a mitigation of the stress status (Tiwari et al. 2016; Arroussi et al. 2018; Evelin et al. 2019). Similarly, $H_2O_2$ contents drastically increased when lettuce plants were exposed to salinity, but this increase was also attenuated by the presence of the cyanobacterium. In agreement, in *Vitis* plants inoculated with the PGPR *Burkholderia phytofirmans* the $H_2O_2$ production was significantly higher in non-inoculated plantlets than in the bacterized plantlets, under cold stress conditions (Theocharis et al. 2012). It is well known that $H_2O_2$ increases considerably in plants under salinity stress (Gill and Tuteja 2010; Smirnoff and Arnaud 2019).

Although deleterious, this molecule plays a crucial role inducing stress-signaling pathways to inhibit further damages and activating some defensive mechanisms (Yang and Guo 2018; Nephali et al. 2020). Interestingly, here we observed that, under standard conditions, the presence *O. lusitanica* enhanced both proline and $H_2O_2$ production in the leaves of lettuce plants. These results probably emphasize the priming effect of the cyanobacterium (Mauch-Mani et al. 2017), promoting the production of antioxidants and therefore improving the defensive capacity of the plants to resist stress. This defense priming triggered by the cyanobacterium further involves an increase in GSH production, a low- molecular weight thiol with functions in detoxification and redox turnover and active in defense signal transduction cascades (Noctor et al. 2012). Similarly, *Bacillus amyloliquefaciens* helps maize plants to tolerate salt stress by increasing glutathione content, however, it does not enhance GSH levels under standard conditions (Chen et al. 2016).

MDA levels reflect the integrity of membranes and it also acts as a signal that triggers defensive mechanisms (Alché 2019). As it was foreseeable, MDA contents drastically increased in lettuce plants exposed to salinity stress, both in presence and absence of *O. lusitanica*, although less pronounced in the former condition. Similar reduction in MDA accumulation was observed in plants under drought and salt stress after inoculation with PGPRs and arbuscular mycorrizal, implying the protective effect of these beneficial microorganisms (Tiwari et al. 2016; Santander et al. 2019).

Plants are also equipped with an enzymatic defense system (Gill and Tuteja 2010; Soltabayeva et al. 2021) that can be activated by beneficial microorganisms helping plants to cope with stresses (Bharti et al. 2016; Habib et al. 2016). However, in this study, none of the enzymes evaluated (CAT, SOD, APX) was elicited due to the application of the cyanobacterium, and only CAT activity increased under salinity stress. In agreement, a recent work shows that lettuce plants under salt stress do not display an increase in the enzymatic antioxidant defense system (Santander et al. 2019). However, these authors showed that the inoculation of the plants with arbuscular mycorrizal fungal promotes stress tolerance by triggering an increase in SOD and CAT activities. Likewise, the activity of GDH, an enzyme of the nitrogen metabolism that can be induced by stress, did not increase under the salinity stress conditions of this study. However, GDH as well as GS and NR activities increased in the presence of the cyanobacterium, suggesting that the presence of *O. lusitanica* is able to stimulate nitrogen assimilation despite not having a significant effect on plant growth. Improvement in plant nutrition induced by non-nitrogen fixing cyanobacteria was widely reported (Li et al. 2019; Omoarelojie et al. 2021; Poveda 2021), and recently, the biostimulatory effect of several microalgal polysaccharides on the growth of tomato plants was assigned to the increase in the activity of nitrogen-related enzymes as NR and GDH (Rachidi et al. 2020).

In conclusion, the inoculation of lettuce plants with the cyanobacterium *O. lusitanica* activates their non-enzymatic antioxidant defenses, thus increasing the plants resilience to salinity stress. Priming effect of *O. lusitanica* in lettuce plants may occur, eliciting the synthesis of proline, $H_2O_2$ and reduced glutathione. These molecules can act as signals potentiating the protective responsiveness of the plants exposed to salinity stress. Furthermore, changes in the nitrogen assimilation pathway are induced in lettuce plants by the cyanobacterium, enhancing the activity of several nitrogen metabolism enzymes, although not significantly affecting biometric parameters in the growth conditions tested.

Currently, agricultural strategies employed to mitigate abiotic stresses are scarce; therefore, biostimulants, non-nutrient substances or microorganisms can be employed to promote plant growth and/or health. Here, we have isolated and described a new terrestrial cyanobacterial species, *Oculatella lusitanica*, with the capacity to boost the resistance of lettuce plants to salinity stress. In addition, we gained a first insight into the plants' physiological response, triggered by the cyanobacterium and leading to higher stress tolerance. This native microorganism can be a valuable tool for sustainable agricultural practices and land recovery, namely in coastal areas of northern Portugal, where lettuces are grown on sandy soils with high salinity.

**Data availability** Data that support the findings of this study have been deposited in GenBank with the MN06191 accession code (https://www.ncbi.nlm.nih.gov/nuccore/MN061918). The authors declare that all the other data supporting the findings of this study are available within the article and its supplementary information file.

## Declarations

**Conflict of interest statement** The authors declare that they have no conflict of interest.

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