



## Research article

Impact of soybean-associated plant growth-promoting bacteria on plant growth modulation under alkaline soil conditions<sup>☆</sup>Mariana Roriz<sup>a,\*</sup>, Sofia I.A. Pereira<sup>a</sup>, Paula M.L. Castro<sup>a</sup>, Susana M.P. Carvalho<sup>b</sup>, Marta W. Vasconcelos<sup>a</sup><sup>a</sup> Universidade Católica Portuguesa, CBQF – Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Rua Diogo Botelho 1327, 4169-005, Porto, Portugal<sup>b</sup> GreenUPorto – Research Centre on Sustainable Agrifood Production / Inov4Agro & DGAOT, Faculty of Sciences, University of Porto, Campus de Vairão, Rua da Agrária 747, 4485-646, Vairão, Portugal

## ARTICLE INFO

## Keywords:

Biofertilizer  
Calcareous soil  
Fe-related genes  
Glycine max  
Photosynthetic parameters  
Fe-deficiency

## ABSTRACT

Conventional strategies to manage iron (Fe) deficiency still present drawbacks, and more eco-sustainable solutions are needed. Knowledge on soybean-specific diversity and functional traits of their plant growth-promoting bacteria (PGPB) potentiates their applicability as bioinoculants to foster soybean performance under calcareous soil conditions. This work aimed to assess the efficacy of PGPB, retrieved from soybean tissues/rhizosphere, in enhancing plant growth and development as well as crop yield under alkaline soil conditions.

Seventy-six bacterial strains were isolated from shoots (18%), roots (53%), and rhizosphere (29%) of soybean. Twenty-nine genera were identified, with *Bacillus* and *Microbacterium* being the most predominant. Based on distinct plant growth-promoting traits, the endophyte *Bacillus licheniformis* P2.3 and the rhizobacteria *Bacillus aerius* S2.14 were selected as bioinoculants.

*In vivo* tests showed that soybean photosynthetic parameters, chlorophyll content, total fresh weight, and Fe concentrations were not significantly affected by bioinoculation. However, inoculation with *B. licheniformis* P2.3 increased pod number (33%) and the expression of Fe-related genes (FRO2, IRT1, F6'H1, bHLH38, and FER4), and decreased FC-R activity (45%). Moreover, bioinoculation significantly affected Mn, Zn, and Ca accumulation in plant tissues.

Soybean harbors several bacterial strains in their tissues and in the rhizosphere with capacities related to Fe nutrition and plant growth promotion. The strain *B. licheniformis* P2.3 showed the best potential to be incorporated in bioinoculant formulations for enhancing soybean performance under alkaline soil conditions.

## 1. Introduction

Legumes grown in calcareous soils, under alkaline conditions (pH 7.5 to 8.5), usually suffer from iron (Fe) deficiency due to the limited bioavailability of this micronutrient under such conditions [1]. This leads to the appearance of iron deficiency chlorosis (IDC), characterized with the plants exhibiting symptoms such as yellowing and interveinal chlorosis of the young leaves, and stunted growth

<sup>☆</sup> Genetic enhancement and advances in crop breeding.

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<https://doi.org/10.1016/j.heliyon.2023.e14620>

Received 30 September 2022; Received in revised form 12 March 2023; Accepted 13 March 2023

Available online 18 April 2023

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[2], severely affecting plants' yield and quality [3–6]. Moreover, the reduced chlorophyll content of young leaves leads to a decrease in the photosynthetic rate, stomatal conductance, and transpiration rate [7].

Plants employ distinct mechanisms to efficiently uptake Fe from soils. Soybean uses a reduction-based strategy (Strategy I) (Fig. 1), which involves rhizosphere acidification and activation of the ferric chelate reductase (FC-R), with expression of the ferric reductase oxidase 2 (FRO2) which reduces  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$ . The soluble Fe is then imported into the root epidermal cells by the iron-regulated transporter 1 (IRT1) which is induced under Fe-limited conditions [8,9]. It was found that several other compounds play important roles in the reduction step conducted by FRO2, such as organic acids, flavins, phenolics, and sugars [10,11]. These compounds are regulated by specific genes, such as feruloyl-CoA 6'-hydroxylase (F6'H1) which controls the synthesis and release of phenolic compounds (e.g. coumarins) that bind and reduce  $\text{Fe}^{3+}$ , improving Fe uptake under limiting conditions [12–15]. The basic helix-loop-helix protein 38 (bHLH38) is also involved in the regulation of Fe uptake in Strategy I plants [16,17], while Fe storage is under the control of ferritin proteins, which also protect cells from oxidative stress [18] and play a role in the root system architecture [19]. There are four ferritin genes (FER1, FER2, FER3, and FER4), with the latter two being expressed in the leaves [20].

The search for more sustainable approaches to manage Fe deficiency is essential, as the existing ones are costly and environmentally unfriendly [21].

The knowledge of the multiplicity of plant-associated bacteria is of extreme importance as it allows the understanding of their ecological role and potential for biotechnological application in agriculture, including the management of crop performance under alkaline soil conditions. Previous studies have shown that *Acinetobacter*, *Bacillus*, *Enterobacter*, *Microbacterium*, *Paenibacillus*, *Pseudomonas*, and *Stenotrophomonas* are amongst the predominant genera with plant growth-promoting potential associated with soybean plants [22–30].

Plant growth-promoting bacteria (PGPB) can modulate the uptake and accumulation of several nutrients (e.g. Fe, zinc (Zn), manganese (Mn), magnesium (Mg), calcium (Ca), phosphorus (P), and potassium (K)) in two different ways: by increasing nutrient availability, and/or improving plant access to nutrients [31]. PGPB may potentiate Fe uptake and plant growth by: i) increasing Fe solubilization, through the production of organic acids, phenolic compounds, chelating agents, and siderophores; ii) overexpressing FRO2 and IRT1 genes, triggering Fe-deficiency signaling pathways; iii) improving photosynthetic capacity through the indirect increase of chlorophyll content; iv) synthesizing indole-3-acetic acid (IAA) which may contribute to the development of root system, improving nutrient uptake [32–36] increasing the activity of the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase. This enzyme decreases the ethylene levels in plants, reducing its negative impact on plant physiological processes [37], having extreme importance when plants are exposed to stress [38], such as alkaline conditions. The role of PGPB on Fe uptake and accumulation has been demonstrated in recent works with legumes [39–43].

The diversity of rhizo- and endophytic bacteria on soybean has been studied, however, most works focus on rhizobia isolated from root nodules, and to the best of our knowledge, so far, no work has explored the PGPB potential to improve legumes' performance under alkaline soil conditions. In a previous study, we have shown that two bacterial endophytes, isolated from maize tissues, with good plant growth-promoting traits and Fe-uptake abilities were able to improve Fe nutrition in 21 days old soybean plants grown in a calcareous soil [43]. The present study intends to go one-step further by testing the hypothesis that native PGPB have a significant effect on soybean plants at full maturity stage, enhancing crop yield under alkaline soil conditions. In this work our goal was to evaluate the potential of PGPB isolated from soybean tissues/rhizosphere in the promotion of plant growth and development and in soybean yield under alkaline soil conditions at the V3 growth stage and at full maturity. To this end, PGPB from soybean tissues and

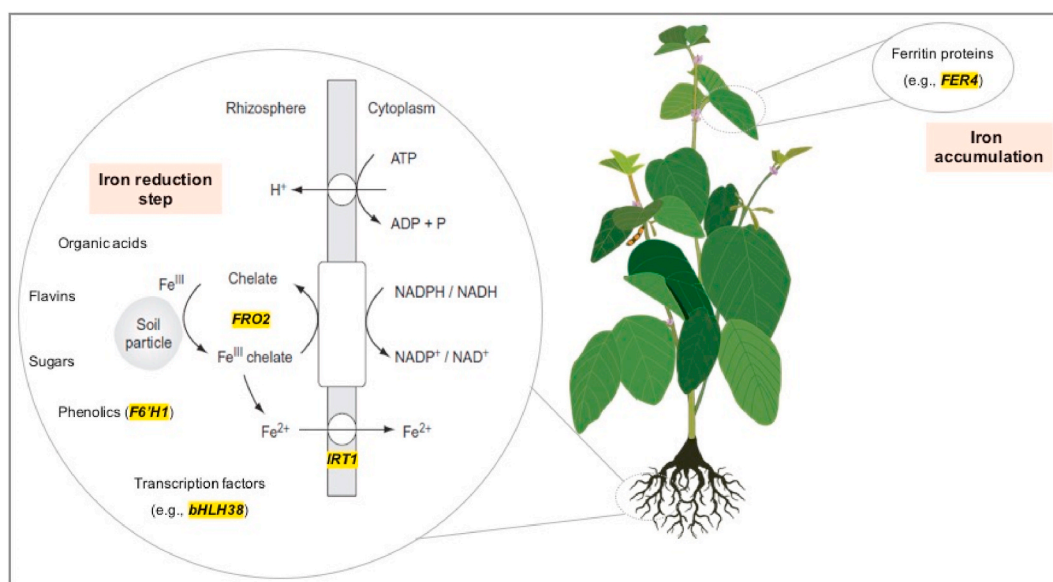


Fig. 1. Schematic representation of the main molecular mechanisms involved in iron uptake and accumulation in soybean.

rhizosphere were isolated and characterized. Thereafter, the best-performing isolates were applied *in vivo*. Assuming that PGPB inoculation has a positive effect on the nutritional status and yield, and on the physiological and molecular plant response to Fe limitation, we assessed the photosynthetic parameters, chlorophyll content, total fresh weight (FW), plant height, root length, FC-R activity, mineral quantification, and gene expression (V3 growth stage) and dry weight (DW), no. Of pods, and seeds per pod (full maturity).

## 2. Materials and methods

### 2.1. Isolation of PGPB

Four healthy soybean plants cv. PI 635039 and their rhizospheric soil were sampled at random from an agricultural soil in Retorta (Vila do Conde, Portugal; 41,349,612, -8.719009). For the isolation of bacterial endophytes, plant surface was sterilized according to Luo et al. [44] with some modifications: a first wash with tap water was done followed by three rinses with deionized water. After separation into shoots and roots, plant tissues were dipped for 2 min in 75% (v/v) ethanol and then 2 min in 25% (v/v) commercial bleach; final washes with deionized sterile water were made to remove the sterilization agents. To achieve the success of surface disinfection process, 100  $\mu$ L of water from the final rinse was spread onto Trypticase Soy Agar (TSA; Liofilchem, Italy) medium and incubated for 3 days at 30 °C. No bacterial growth was found. One gram of each plant tissue (shoot and root) was macerated with 9 mL of sterile phosphate-buffered saline solution (PBS, g/L: Na<sub>2</sub>HPO<sub>4</sub>, 1.44; KH<sub>2</sub>PO<sub>4</sub>, 0.24; KCl, 0.20; NaCl, 8.00; pH 7.4) with a mortar and pestle under sterile conditions. Serial dilutions were prepared in 0.85% (w/v) saline solution; 100  $\mu$ L of each dilution were plated, in duplicate, on TSA medium supplemented with 10 mg/L of fungicidin (Amresco, USA) after autoclaving, and incubated at 30 °C for 7 days. For the isolation of rhizobacteria, 1 g of fresh rhizospheric soil was mixed with 9 mL of 0.85% (w/v) saline solution and with 2 drops of Tween 20 (Sigma-Aldrich, USA) and vortexed for 10 min. Serial dilutions were prepared in 0.85% (w/v) sterile saline solution and 100  $\mu$ L of each dilution were plated, in duplicate, on TSA medium and incubated at 30 °C for up to 10 days. Bacterial isolates from plant tissues and soil were daily monitored and selected based on their morphology and color. They were further purified by sub-culturing on TSA medium.

### 2.2. Identification of bacterial isolates

Genomic DNA was obtained by the heat-shock extraction method [45]. Random Amplification of Polymorphic DNA (RAPD) analysis was used as a primary method to group the isolates. Amplification reactions (25  $\mu$ L) were performed as follows: 12.5  $\mu$ L of NZYtaq II 2 $\times$  Green Master Mix (Nzytech, Lisbon, Portugal), 1  $\mu$ L of primer M13 (5'-GAGGGTGGCGGT TCT-3') (MWG-Biotech), and 2.5  $\mu$ L of sample DNA. A negative control was included for each PCR reaction. Amplification was performed in a thermocycler DOPPIO (VWR, USA) programmed for an initial denaturation step at 94 °C for 5 min, followed by 45 cycles of 1 min at 94 °C, 2 min at 34 °C and 2 min at 72 °C, and a final extension step at 72 °C for 10 min. PCR products were analyzed on a 1.5% agarose gel stained with GelRed (Biotium, USA) for 135 min at 80 V. RAPD patterns were compared using Bionumerics software (Applied Maths, St- Martens-Laten, Belgium) and clustered according to their similarities. Band matching position tolerance was set at 1%. Seventy-three different profiles were recognized after RAPD analysis.

Partial sequence of the 16 S ribosomal RNA (rRNA) gene was carried out using the universal primers 27 F (5'-GAGTTT-GATCCTGGCTCAG-3') and 1492 R (5'-ACCTTGTTACGACTT-3') (MWG-Biotech). PCR amplifications were performed in a 25  $\mu$ L-reaction mixture containing 12.5  $\mu$ L of NZYtaq II 2 $\times$  Green Master Mix (Nzytech, Lisbon, Portugal), 0.25  $\mu$ L of each primer, and 8  $\mu$ L of DNA template. The cycling procedure was performed using an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were sent for purification and sequencing to Macrogen Inc. (Seoul, Republic of Korea). Sequence editing and inspection were performed using BioEdit program 7.0. The 16 S rRNA sequences of the bacterial strains with quality were deposited in GenBank database under the accession numbers MW414477-MW414500, MW544871, and MW544872. The selection of the isolates for further testing excluded those considered human or plant pathogens.

### 2.3. Screening for Fe nutrition-related traits

Bacterial strains were tested for their ability to grow in TSB medium with pH adjusted to 9.0, to reduce Fe<sup>3+</sup>, and to produce organic acids as described before Roriz et al. [43]. For each analysis, experiments were repeated three times for each bacterial strain and OD determinations were made in triplicate. A growth inhibition percentage lower than 70% was defined as an exclusion criterion for the greenhouse experiment.

### 2.4. Screening for plant growth-promoting traits

Production of siderophores by bacterial strains was evaluated using a chrome azurol S (CAS) agar medium as described by Schwyn and Neilands [46]. Orange halos around the colonies on CAS agar were indicative of siderophore excretion. Three replicates were made for each bacterial strain.

The amount of IAA produced by bacterial isolates was determined according to the method of Gordon and Weber [47]. Briefly, an aliquot of 500  $\mu$ L of the supernatant obtained from bacterial cultures grown in the presence of L-tryptophan (1%) was mixed with 350

$\mu\text{L}$  of Salper reagent. The absorbance of pink color developed after 30 min incubation in dark was read at 530 nm. The IAA concentration was determined using a calibration curve (0–80  $\mu\text{g}/\text{mL}$ ) of pure IAA as a standard. The experiments were repeated three times, and IAA determinations were made in triplicate.

The ACC deaminase activity of cell-free extracts was determined by estimating the amount of  $\alpha$ -ketobutyrate generated by the enzymatic hydrolysis of ACC [48] according to the procedure of Honma and Shimomura [49]. The experiments were repeated three times, and determinations were made in triplicate.

## 2.5. Greenhouse experimental design

The endophyte and the rhizobacteria identified in this study as *Bacillus licheniformis* P2.3 and *Bacillus aerius* S2.14, respectively, were selected for the pot greenhouse assay. The choice of these strains relied on distinct characteristics related to their ability to reduce  $\text{Fe}^{3+}$ , to produce siderophores, organic acids, IAA, and ACC-deaminase activity.

The greenhouse pot experiment consisted of a factorial design with three treatments: i) Control – without inoculation; ii) inoculation with *B. licheniformis* P2.3; and iii) inoculation with *B. aerius* S2.14. Each treatment was replicated 12 times. Seeds of *Glycine max* L. cultivar Williams 82 were disinfected using 70% (v/v) ethanol for 5 min, followed by a solution of 1.2% (v/v) sodium hypochlorite and 0.02% (w/v) sodium dodecyl sulphate (SDS) for 15 min. Seeds were then rinsed five times in sterile water. Germination was performed in filter paper for seven days in the dark, at 25 °C.

One seedling was transferred to pots (3 L) containing 150 g of a lower bed of light expanded clay aggregate (LECA®) and 2 kg of an upper bed of sieved (<2 mm) calcareous agricultural soil (pH 8.2) collected in the southern part of Portugal. The physicochemical properties of the soil are presented in Table 1. Soil was not autoclaved to better mimic real-life conditions to which plants would be exposed in a future agricultural setting.

For the inoculation, bacterial strains were grown overnight in TSB medium at 30 °C. Cells in the exponential phase were harvested by centrifugation at 5000 rpm for 10 min, washed twice with sterile saline solution (0.85% NaCl), and centrifuged again. Bacterial inoculum was prepared by resuspending pellet in sterile saline solution to get an inoculum density of ca.  $10^8$  CFU/mL. Bacterial suspensions (50 mL/pot) were poured into the substrate surface at the time of seedling transplantation. Sterile saline solution (50 mL/pot) was added to control. Soybean plants grown in a commercial substrate were used as border plants and pots with treated plants were randomly distributed and watered daily with tap water through an automatic irrigation system.

**Table 1**

Percentage of growth inhibition at pH 9.0, concentration of reduced  $\text{Fe}^{2+}$ , and production of siderophores, organic acids, indole-3-acetic acid (IAA), and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity of bacterial strains. Data are means  $\pm$  SEM (n = 9).

Isolate	% inhibition (pH 9.0)	Reduced $\text{Fe}^{2+}$ (mM)	Siderophores	Organic acids	IAA (mg/L)	ACC (nmol/g/h)
P1.2	40 $\pm$ 3	0.047 $\pm$ 0.001	++	–	1.089 $\pm$ 0.145	1.206 $\pm$ 0.119
P1.3	–39 $\pm$ 1	0.038 $\pm$ 0.001	++	–	0.849 $\pm$ 0.194	1.481 $\pm$ 0.662
P1.5	–812 $\pm$ 2	0.036 $\pm$ 0.001	++	–	2.185 $\pm$ 0.339	2.610 $\pm$ 0.447
P2.3	–105 $\pm$ 2	0.035 $\pm$ 0.001	+	+	2.082 $\pm$ 0.194	5.469 $\pm$ 0.297
P3.1	14 $\pm$ 4	0.026 $\pm$ 0.001	++	–	4.171 $\pm$ 0.145	7.591 $\pm$ 0.264
P3.3	45 $\pm$ 5	0.033 $\pm$ 0.001	++	–	2.151 $\pm$ 0.022	12.467 $\pm$ 0.488
P4.1	96 $\pm$ 1	0.035 $\pm$ 0.001	++	+	2.253 $\pm$ 0.436	1.455 $\pm$ 0.651
R1.2	–27 $\pm$ 4	0.038 $\pm$ 0.001	+	–	2.014 $\pm$ 0.053	11.118 $\pm$ 0.547
R1.4	38 $\pm$ 6	0.040 $\pm$ 0.000	+	–	2.082 $\pm$ 0.291	4.273 $\pm$ 0.417
R1.9	60 $\pm$ 0	0.040 $\pm$ 0.001	++	–	2.562 $\pm$ 0.291	3.269 $\pm$ 0.266
R1.10	97 $\pm$ 1	0.029 $\pm$ 0.001	+++	–	3.966 $\pm$ 0.145	7.263 $\pm$ 0.119
R2.1	58 $\pm$ 3	0.031 $\pm$ 0.001	++	–	2.767 $\pm$ 0.090	7.989 $\pm$ 0.876
R2.2	3 $\pm$ 4	0.038 $\pm$ 0.000	++	–	3.452 $\pm$ 0.291	4.161 $\pm$ 0.101
R2.14	85 $\pm$ 3	0.029 $\pm$ 0.000	++	–	2.836 $\pm$ 0.097	2.317 $\pm$ 0.088
R3.3	31 $\pm$ 1	0.030 $\pm$ 0.001	++	–	2.288 $\pm$ 0.102	1.552 $\pm$ 0.101
R3.7	–231 $\pm$ 3	0.038 $\pm$ 0.001	++	–	2.288 $\pm$ 0.029	3.266 $\pm$ 0.117
R4.3	32 $\pm$ 5	0.055 $\pm$ 0.001	+++	–	13.144 $\pm$ 0.048	8.213 $\pm$ 0.118
R4.8	22 $\pm$ 6	0.041 $\pm$ 0.001	++	+	11.979 $\pm$ 0.533	10.070 $\pm$ 0.269
R4.12	37 $\pm$ 5	0.048 $\pm$ 0.001	++	–	7.699 $\pm$ 0.097	3.840 $\pm$ 0.118
S1.1	–327 $\pm$ 4	0.036 $\pm$ 0.001	+	–	2.493 $\pm$ 0.048	4.103 $\pm$ 0.235
S1.3	37 $\pm$ 5	0.031 $\pm$ 0.001	++	–	9.377 $\pm$ 0.048	8.890 $\pm$ 0.288
S2.2	16 $\pm$ 3	0.033 $\pm$ 0.001	++	–	12.527 $\pm$ 0.145	8.917 $\pm$ 0.128
S2.3	30 $\pm$ 4	0.030 $\pm$ 0.001	++	–	8.521 $\pm$ 0.387	7.275 $\pm$ 0.288
S2.4	–73 $\pm$ 0	0.023 $\pm$ 0.000	++	–	7.288 $\pm$ 0.581	2.746 $\pm$ 0.134
S2.6	24 $\pm$ 3	0.025 $\pm$ 0.001	+	–	9.103 $\pm$ 0.048	3.865 $\pm$ 0.138
S2.10	17 $\pm$ 6	0.037 $\pm$ 0.001	+++	–	13.760 $\pm$ 0.726	4.235 $\pm$ 0.130
S2.14	–48 $\pm$ 6	0.017 $\pm$ 0.001	+++	–	9.514 $\pm$ 0.242	8.750 $\pm$ 0.108
S3.4	25 $\pm$ 4	0.032 $\pm$ 0.001	++	–	16.568 $\pm$ 0.145	5.050 $\pm$ 0.147
S4.1	6 $\pm$ 2	0.025 $\pm$ 0.001	+	–	4.103 $\pm$ 0.145	9.069 $\pm$ 0.153

–, negative.

+, positive/weak.

++, intermediate.

+++ , strong.

Plants were collected in two phases: i) 24 days after inoculation (V3 growth stage), half of the plants were collected and analyzed for photosynthetic parameters (using an InfraRed Gas Analyzer (IRGA)), chlorophyll content (using a Soil Plant Analysis Development (SPAD) chlorophyll meter), total FW, plant height, root length, FC-R activity, mineral quantification, and gene expression; ii) 128 days after inoculation (full maturity), the remaining plants were collected and analyzed for DW, no. Of pods, and seeds per pod.

#### 2.5.1. IRGA analysis

The photosynthetic rate ( $\mu\text{mol CO}_2/\text{m}^2/\text{s}$ ), stomatal conductance ( $\text{mol H}_2\text{O}/\text{m}^2/\text{s}$ ), and transpiration rate ( $\text{mol}/\text{m}^2/\text{s}$ ) were measured in the youngest trifoliolate leaf of six independent plants 24 days after inoculation, using an IRGA LI-6400 portable photosynthesis system (LI-COR Inc., Lincoln, NE, USA).

#### 2.5.2. SPAD and plant biometrical parameters

Twenty-four days after inoculation, leaf chlorosis was assessed using a SPAD chlorophyll meter (Konica Minolta SPAD-502 Plus; Minolta, Osaka, Japan) in the youngest trifoliolate leaf of six independent plants. Plant height and root length were measured, and FW was determined after separation into trifoliate leaves and roots. At the end of the experiment (128 days), the no. Of pods and seeds per pod were determined for the other six plants of each treatment, as well as DW after drying plant tissues at 60 °C.

#### 2.5.3. -R activity measurements

Twenty-four days after inoculation, root Fe reductase was quantified in six independent plants as described by Roriz et al. [43].

#### 2.5.4. Microwave-assisted digestion and ICP analysis

Dried plant tissues (trifoliate leaves and roots;  $n = 3$ ) were digested through microwave-assisted digestion and the concentration of Fe, Zn, Mn, Mg, Ca, P, and K was analyzed as described before in Roriz et al. [43]. All analyses per biological replicate were done in triplicate.

#### 2.5.5. rRNA extraction and cDNA synthesis

rRNA extraction and cDNA synthesis were performed in three biological replicates from each treatment according to Roriz et al. [43].

#### 2.5.6. Primer design and qRT-PCR

The transcript levels of FRO2, IRT1, F6'H1, bHLH38, and FER4 genes were analyzed, using 18 S rRNA and ubiquitin as reference genes, as described before [43]. FER4 expression is specific to the leaves and thus its expression was only evaluated in trifoliate leaves. Three biological replicates were analyzed, and two technical repetitions of each biological replicate were performed. The primer pairs used for the qPCR analysis are listed in [Supplementary Table 1](#).

### 2.6. Physicochemical soil properties

The physicochemical properties of the soil (pH, organic content, total  $\text{CaCO}_2$ , extractable K, P, B, Cu, Zn, Fe, and Mn, electrical conductivity, and total nitrogen (N)) were evaluated in samples collected at the beginning, before inoculation with bacteria (day 1), and at the end of the greenhouse experiment (day 128). For this purpose, for each treatment, we analyzed a 3-sample pool of the rhizospheric region.

### 2.7. Statistical analysis

Data were subjected to analysis of variance (one-way ANOVA) for the effect of bacterial inoculation using the GraphPad Prism version 7.0 (San Diego, CA, USA). Significant differences between treatments were determined using Tukey's test ( $P < 0.05$ ).

### 2.8. Soil analysis

The characterization of rhizospheric soil was performed at the beginning of the experiment, before inoculation with PGPB (day 1), and at final harvest (day 128) ([Table 1](#)). In general, organic matter content tended to be higher at the end of the experiment, especially in soil inoculated with the strain *B. licheniformis* P2.3. Regarding nutrients, the concentration of boron (B), Zn, and N tended to be higher at the end of the experiment, while an opposite trend was observed for P, copper (Cu), Fe, and Mn.

## 3. Results

### 3.1. Isolated from soybean plants

Overall, based on the distinct colony characteristics, a total of 76 bacterial strains were isolated from shoots (18%), roots (53%), and rhizosphere (29%) of soybean plants. After RAPD analysis, 73 different profiles were recognized. Based on the 16 S rRNA gene sequencing, 51 isolates were identified and 29 genera were detected ([Supplementary Table 2](#)). However, many of the sequences obtained showed poor quality, and so bacterial identification associated with those sequences should be interpreted carefully.



*Bacillus* and *Microbacterium* were the most common genera followed by *Paenibacillus* and *Stenotrophomonas*. Strains *Staphylococcus epidermidis* P2.2, *Achromobacter pulmonis* P3.4, *Staphylococcus xylosum* P.3.5, *Bacillus oleronius* P3.6, *Rheinheimera mesophila* R1.1, *Bosea thiooxidans* R1.5, *Sphingomonas faucium* R1.12, *Stenotrophomonas pavanii* R1.13, *Dyadobacter jiangsuensis* R1.15, *Microbacter margulisiae* R2.5, *Brachybacterium faecium* R2.12, *Flexithrix dorotheae* R3.2, *Bacteroides fragilis* R3.4, *Sphingobacterium chuzhouense* R3.11, *Microbacterium jejuense* R4.2, *Luteimonas aestuarii* R4.4, *Vitreoscilla stercoraria* R4.7, *Staphylococcus warneri* R4.13, *Bacillus licheniformis* S1.6, *Bacillus cereus* S1.7, *Arthrobacter humicola* S2.8, and *Fictibacillus enclensis* S4.2 were excluded from the subsequent assays as they are considered human or plant pathogens.

### 3.2. Fe reduction ability and plant growth promoting traits of selected PGPB

The ability of bacterial strains to grow at high pH values, to reduce  $\text{Fe}^{3+}$ , to produce organic acids, siderophores, and IAA, and the ACC-deaminase activity are presented in Table 2. Most bacterial strains (90%) showed growth inhibition at pH 9.0 lower than 70% (the value above which we defined as an exclusion criterion for the greenhouse experiment). The ability to reduce  $\text{Fe}^{3+}$  was evaluated by the produced amount of  $\text{Fe}^{2+}$ . Concentrations of  $\text{Fe}^{2+}$  ranged from 0.017 to 0.055 mM and 76% of the isolates produced an amount of reduced  $\text{Fe}^{2+}$  above 0.030 mM. The mean value of reduced  $\text{Fe}^{2+}$  was significantly higher in root endophytes (0.038 mM) if compared with rhizospheric strains (0.029 mM). All tested strains produced siderophores, with 66% showing intermediate production, while 14% showed strong production. Production of organic acids was only observed in 10% of the bacterial isolates. IAA production was detected in all bacterial isolates in the presence of 1% of tryptophan after 48 h of incubation. The levels of IAA produced by PGPB ranged from 0.849 to 16.568 mg/L. The mean value was significantly higher in rhizobacteria (9.325 mg/L) compared with root (4.756 mg/L) and shoot (2.111 mg/L) endophytes. ACC-deaminase activity was detected in all bacterial strains with values ranging from 1.206 to 12.467 nmol  $\alpha$ -ketobutyrate/g/h.

Two promising strains, *B. licheniformis* P2.3 and *B. aerius* S2.14, were selected for pot experiments based on their distinct PGP characteristics. The strain P2.3 is a shoot endophyte that showed increased ability to reduce  $\text{Fe}^{3+}$  and to produce IAA, tested positive for the production of organic acids, but showed weak production of siderophores and low ACC-deaminase activity; the strain S2.14 was isolated from the rhizosphere, showed strong production of siderophores, moderate IAA synthesis and ACC-deaminase activity, low ability to reduce  $\text{Fe}^{3+}$ , and tested negative for the production of organic acids.

### 3.3. Influence of PGPB inoculation in soybean growth, photosynthetic activity, and Fe nutrition

Bacterial inoculation did not significantly ( $P > 0.05$ ) influence the photosynthetic and transpiration rate, and the stomatal conductance of soybean plants (Fig. 2). After 40 days no significant differences were observed between treatments for SPAD and FW measurements (Table 3). However, plants inoculated with the strain *B. licheniformis* P2.3 showed higher height (+23%) than plants inoculated with the strain *B. aerius* S2.14, despite no significant differences were observed in relation to control. Plants inoculated with the strain S2.14 showed a significant decrease (25%) in root length compared with control. Similarly, at full maturity (after 128 days of inoculation) no significant differences were observed between treatments for DW and seeds per pod (Table 3). However, plants inoculated with the strain *B. licheniformis* P2.3 showed an increase of 33% in the no. Of pods compared with control.

The concentration of micro- (Fe, Mn, Zn) and macronutrients (Ca, K, Mg, P) in the trifoliates and roots of *G. max* plants is presented in Table 4. No significant differences were observed in the concentration of Fe in the trifoliates and roots of inoculated plants compared with control, while Mn concentration was significantly decreased in the trifoliates of inoculated plants compared with non-inoculated plants. The concentration of Zn in roots of plants inoculated with the strain *B. licheniformis* P2.3 was increased by 62% compared with control plants. Likewise, a significant increase (20%) was verified for Ca concentration in trifoliates. No significant differences in the concentration of K, Mg, and P in plant tissues were observed between treatments.

The activity of the Fe reductase in the roots of soybean plants is shown in Fig. 3. A significant decrease in FC-R activity was verified in plants inoculated with the strains.

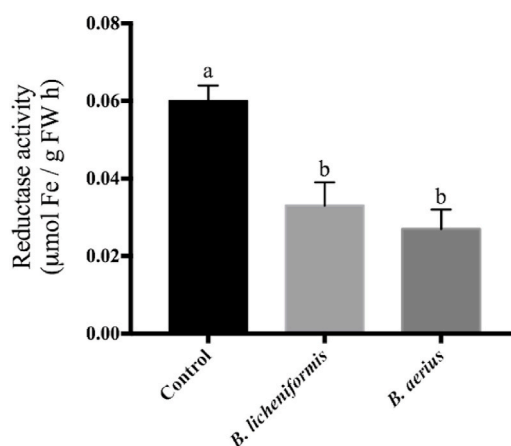
P0.20.3 (45%) and S2.14 (55%) compared with non-inoculated plants.

The expression of five known genes associated with Fe nutrition studied using qPCR included: FRO2, IRT1, F6'H1, and bHLH38 in roots, as well as FER4 in trifoliates. In general, gene expression increased in inoculated plants (Fig. 4). FRO2 expression increased by

**Table 2**

SPAD values and morphological parameters of soybean plants 24 days after inoculation and dry weight, no. Of pods and seeds per pod 128 days after inoculation with saline solution (Control), *B. licheniformis* P2.3, and *B. aerius* S2.14. Data are mean  $\pm$  SEM of six biological replicates. Different letters indicate significant differences ( $P < 0.05$ ) between treatments. Statistical analysis was performed independently for each analysis and time point.

Time point	Parameter	Control	<i>B. licheniformis</i>	<i>B. aerius</i>
24 days after inoculation	SPAD	33.12 $\pm$ 2.18 <sup>a</sup>	29.48 $\pm$ 2.44 <sup>a</sup>	32.67 $\pm$ 1.61 <sup>a</sup>
	Fresh weight (g)	5.22 $\pm$ 0.82 <sup>a</sup>	5.81 $\pm$ 0.67 <sup>a</sup>	5.56 $\pm$ 0.59 <sup>a</sup>
	Plant height (cm)	40.08 $\pm$ 1.15 <sup>ab</sup>	43.77 $\pm$ 1.77 <sup>a</sup>	35.67 $\pm$ 1.18 <sup>b</sup>
	Root length (cm)	26.98 $\pm$ 0.92 <sup>a</sup>	30.33 $\pm$ 1.66 <sup>a</sup>	21.60 $\pm$ 1.21 <sup>b</sup>
128 days after inoculation	Dry weight (g)	2.39 $\pm$ 0.23 <sup>a</sup>	3.07 $\pm$ 0.38 <sup>a</sup>	2.75 $\pm$ 0.26 <sup>a</sup>
	No. Of pods	3.6 $\pm$ 0.2 <sup>b</sup>	4.8 $\pm$ 0.4 <sup>a</sup>	3.5 $\pm$ 0.2 <sup>b</sup>
	No. Of seeds per pod	1.6 $\pm$ 0.1 <sup>a</sup>	1.6 $\pm$ 0.2 <sup>a</sup>	1.8 $\pm$ 0.1 <sup>a</sup>



**Fig. 2.** - Photosynthetic rate (A), stomatal conductance (B), and transpiration rate (C) of soybean plants 24 days after inoculation. Control – without inoculation; inoculation with the strain *B. licheniformis* P2.3, and inoculation with the strain *B. aerius* S2.14. Data are mean  $\pm$  SEM of three biological replicates. Different letters indicate significant differences ( $P < 0.05$ ) between treatments.

**Table 3**

Mineral concentration on trifoliates and roots of *G. max* plants, grown under alkaline conditions, 24 days after inoculation with saline solution (Control), *B. licheniformis* P2.3, and *B. aerius* S2.14. Data are mean  $\pm$  SEM of three biological replicates. Statistical analysis was performed independently for each mineral and plant tissue. Different letters indicate significant differences ( $P < 0.05$ ) within the same plant tissue between treatments.

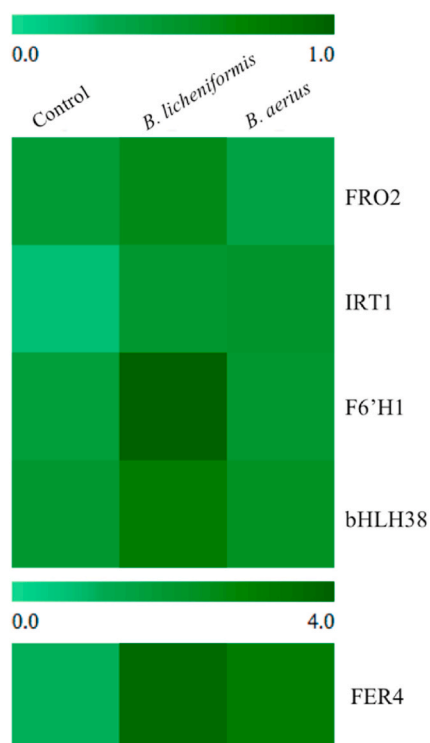
Plant tissue	Treatment	Mineral concentration ( $\mu\text{g/g}$ )						
		Fe	Mn	Zn	Ca	K	Mg	P
Trifoliates	Control	108.5 $\pm$ 15.3 <sup>a</sup>	115.6 $\pm$ 11.1 <sup>a</sup>	13.0 $\pm$ 0.5 <sup>a</sup>	18834.0 $\pm$ 453.3 <sup>b</sup>	7123.5 $\pm$ 879.1 <sup>a</sup>	5151.7 $\pm$ 97.7 <sup>a</sup>	1275.7 $\pm$ 117.2 <sup>a</sup>
	<i>B. licheniformis</i>	89.7 $\pm$ 7.9 <sup>a</sup>	78.4 $\pm$ 4.6 <sup>b</sup>	13.4 $\pm$ 1.0 <sup>a</sup>	22541.6 $\pm$ 232.3 <sup>a</sup>	6480.7 $\pm$ 332.3 <sup>a</sup>	5052.7 $\pm$ 388.8 <sup>a</sup>	1219.9 $\pm$ 102.9 <sup>a</sup>
	<i>B. aerius</i>	102.8 $\pm$ 1.2 <sup>a</sup>	82.2 $\pm$ 3.1 <sup>b</sup>	12.2 $\pm$ 0.6 <sup>a</sup>	20652.3 $\pm$ 950.0 <sup>ab</sup>	7453.5 $\pm$ 463.2 <sup>a</sup>	5323.0 $\pm$ 523.0 <sup>a</sup>	1219.1 $\pm$ 38.1 <sup>a</sup>
Roots	Control	4127.5 $\pm$ 160.4 <sup>A</sup>	98.4 $\pm$ 9.6 <sup>A</sup>	23.3 $\pm$ 0.2 <sup>B</sup>	7822.8 $\pm$ 471.2 <sup>A</sup>	7155.5 $\pm$ 880.8 <sup>A</sup>	7108.5 $\pm$ 692.4 <sup>A</sup>	749.0 $\pm$ 92.6 <sup>A</sup>
	<i>B. licheniformis</i>	4604.5 $\pm$ 485.9 <sup>A</sup>	139.6 $\pm$ 15.8 <sup>A</sup>	37.8 $\pm$ 2.9 <sup>A</sup>	9947.9 $\pm$ 1076.0 <sup>A</sup>	6651.0 $\pm$ 961.0 <sup>A</sup>	7474.8 $\pm$ 773.8 <sup>A</sup>	792.7 $\pm$ 57.1 <sup>A</sup>
	<i>B. aerius</i>	3173.8 $\pm$ 260.9 <sup>A</sup>	99.2 $\pm$ 11.4 <sup>A</sup>	28.8 $\pm$ 1.6 <sup>B</sup>	6917.7 $\pm$ 410.7 <sup>A</sup>	6710.8 $\pm$ 998.7 <sup>A</sup>	6787.2 $\pm$ 373.0 <sup>A</sup>	872.4 $\pm$ 86.6 <sup>A</sup>

38% in the roots of plants inoculated with *B. licheniformis* and decreased by 20% with inoculation with *B. aerius* compared with non-inoculated plants. Expression of IRT1, F6/H1, and bHLH38 genes increased in the roots of plants inoculated with *B. licheniformis* by 135%, 167%, and 59% respectively, while in the roots of plants inoculated with *B. aerius* the expression increased 16%, 183%, and 16% respectively. In trifoliates, a similar trend was observed in the expression of FER4 for the inoculated plants (increases up to 252%).

#### 4. Discussion

In this study, PGPB strains isolated from soybean tissues and rhizosphere showed very good plant growth-promoting traits and ability to reduce  $\text{Fe}^{3+}$ . Bacterial inoculants had a marginal influence in soybean growth; however, the endophytic strain *B. licheniformis* P2.3 increased the number of pods by 33% at full maturity. Despite the fact that bioinoculants decreased FC-R activity (45%), they induced the expression of most of Fe-nutrition related genes (e.g. IRT1, F6/H1, bHLH38, and FER4), suggesting their importance on the regulation of Fe nutrition under alkaline conditions.

The number of bacterial isolates recovered from soybean roots was higher than that obtained from rhizospheric soil, followed by the shoots, which may reflect the intimate contact of radicular system with the rhizosphere during plant growth and development, facilitating the entry of bacteria into the root tissues [50]. The presence of bacterial isolates inside tissues reflects their ability to colonize plant organs, which is a relevant trait for their applicability as inoculants. Several genera were identified indicating a high diversity of culturable bacteria associated with soybean plants. In fact, legumes are known to harbor a remarkable diversity of bacteria in their tissues and rhizosphere, which is probably related to their long history of cultivation and selection under various agroclimatic and geographic conditions [51]. The genera *Bacillus* and *Microbacterium* were the most common followed by genera *Paenibacillus* and *Stenotrophomonas*. These genera have already been associated with soybean plants with enhanced growth abilities [22,24,25,27,30].



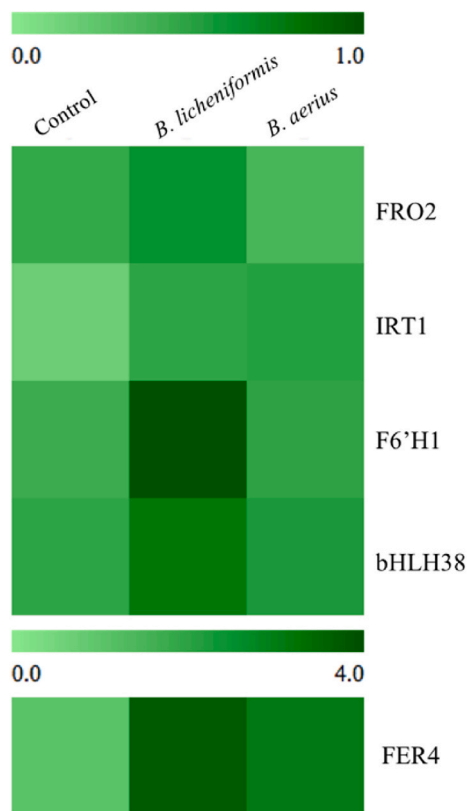
**Fig. 3.** - Root reductase activity of *G. max* plants grown under alkaline conditions. Control – without inoculation; inoculation with the strain *B. licheniformis* P2.3, and inoculation with the strain *B. aerius* S2.14. Data are mean  $\pm$  SEM of six biological replicates. Different letters indicate significant differences ( $P < 0.05$ ) between treatments.

In this study, since the cultivar Williams 82 has an intermediate susceptibility to Fe shortage [52,53] it would be expected that soybean plants showed visual Fe deficiency symptoms, while bioinoculation would contribute to mitigate those negative effects and improve soybean growth and photosynthetic parameters [36,41,54,55]. However, no typical phenotypic symptoms associated to Fe-deficiency were observed in plants, which were confirmed by the biometric and photosynthetic parameters. Similar results were obtained in previous studies [52,56,57]. The absence of symptoms can be explained by the fact that soybean plants were grown in a substrate instead of a hydroponic system where the lack of cation-exchange capacity induces Fe-deficiency symptoms. Moreover, the growth time may also contribute to the observed differences. Several authors have reported the positive influence of PGPB inoculation on plant growth under Fe-deficient conditions [36,41,53,54]. Nonetheless, in the present study, PGPB inoculation did not significantly influence neither soybean growth nor photosynthetic parameters, suggesting that Fe-deficient conditions were not severe enough to induce such effects.

Regarding the plant mineral analysis, overall PGPB inoculation influenced the nutritional level of soybean plants grown under alkaline conditions. Fe concentration was higher in roots than in shoots. Similar results were described by Nagata [55] and Zhou et al. [37] in plants grown under Fe-deficient conditions in a liquid medium or artificial calcareous soil after inoculation with PGPB. Several studies showed that PGPB improve legume growth by enhancing Fe uptake under alkaline conditions [37,41,43]. However, no significant differences were observed in Fe concentration in tissues of inoculated plants compared to non-inoculated plants. It is possible that the Fe-nutrition-related mechanisms promoted by bacteria were insufficient to enhance its accumulation in plant tissues. The FC-R activity is often induced under Fe-limiting conditions to promote Fe uptake [58,59], and PGPB have also been shown to induce FC-R activity under Fe shortage [37,43]. Here, a significant decrease in FC-R activity was observed in plants inoculated with strains *B. licheniformis* (45%) and *B. aerius* (55%) compared to non-inoculated ones, indicating that inoculated plants were under lower Fe deficiency stress and that Fe uptake was regulated through other mechanisms, perhaps at the transcriptional level. Indeed, FRO2 expression increased (38%) in the roots of plants inoculated with the strain *B. licheniformis* P2.3, although this was not reflected in an increase in FC-R activity. The different behavior reported for FC-R activity and FRO2 expression can be related to different regulation timings regarding enzyme activity and gene expression, and because FC-R activity varies with time [60]. It would be interesting to evaluate FC-R activity at different time points to test this hypothesis. Under Fe-limiting conditions, FRO2 expression is usually activated so that  $\text{Fe}^{3+}$  is reduced to  $\text{Fe}^{2+}$  [61]. Zhou et al. [37], Rahimi et al. [62], and Roriz et al. [43] also found an increase in FRO2 expression after inoculation with PGPB under Fe-deficient growth conditions.

The increased expression of IRT1 in inoculated plants suggests that  $\text{Fe}^{2+}$  was effectively collected from the rhizosphere and absorbed by the roots, showing the potential of bacterial isolates on Fe uptake. Similar to FRO2, IRT1 expression was found increased in previous studies with plants inoculated with *B. subtilis* GB03 and *P. polymyxa* BFKC01 [36,63]. F6'H1 and bHLH38 expression also





**Fig. 4.** - Heat map of the expression patterns of FRO2, IRT1, F6'H1, bHLH38, and FER4 genes in *G. max* plants, grown under alkaline conditions. Control – without inoculation; inoculation with the strain *B. licheniformis* P2.3, and inoculation with the strain *B. aerius* S2.14. FRO2, IRT1, F6'H1, and bHLH38 expression was measured in the roots; FER4 expression was measured in the trifoliates. In dark green: increased gene expression; in light green: lower gene expression. Data are means of three independent biological replicates. Corresponding values are presented in [Supplementary Table 3](#).

increased in inoculated plants. These genes are related to FRO2 and IRT1 and are also involved in the improvement of Fe solubilization and uptake under Fe-deficient conditions [64]. FER4 expression increased in the trifoliates of treated plants, suggesting an effective enhancement of Fe uptake and storage [18] in the shoots due to the inoculation, although this was not mirrored later in Fe tissue levels. These findings can support PGPB's role in activating transcriptional mechanisms associated with Fe metabolism.

Inoculation with PGPB also significantly impacted the accumulation of other important minerals (Mn, Zn, and Ca) showing the potential of these inoculants to modulate mineral uptake, which is also important for plant nutrition.

Soil organic matter content tended to be higher at the end of the experiment, especially in soil inoculated with the strain *B. licheniformis* P2.3. This is expected since the residues produced by plants and the bacterial activity can contribute to this increase [65].

Comparing our findings with the results of a previous study of our group Roriz et al. [43], we expect that the selected native soybean isolates, being better adapted to soybean, would show a better performance in the improvement of plant growth and Fe uptake.

## 5. Conclusion

Soybean harbors several bacterial strains in their tissues and the rhizosphere with improved capacities related to Fe nutrition and plant growth promotion. A large fraction of the isolates shows improved capacities to grow at high pH, to reduce  $\text{Fe}^{3+}$ , to produce siderophores, organic acids, IAA, and ACC-deaminase, making them potential candidates for exploitation as future bioinoculants. *B. licheniformis* P2.3 is the most effective in improving soybean yield and performance under alkaline conditions as seen by the increase in the number of pods and expression of Fe-related genes. This is a promising strain to be used in the bioinoculant formulations for improved soybean growth. Other strains identified in this study show untapped potentials with promising Fe related traits and can now be target for future studies.

## Author contribution statement

Mariana Roriz: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Sofia I. A. Pereira: Analyzed and interpreted the data.

Paula M. L. Castro; Susana M. P. Carvalho; Marta W. Vasconcelos: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

## Funding statement

Dr. Mariana Roriz was supported by Fundação para a Ciência e a Tecnologia [SFRH/BD/109432/2015].

This work was supported by Horizon 2020 [727973], Fundação para a Ciência e a Tecnologia [UIDB/05748/2020; UIDB/50016/2020; UIDP/05748/2020].

## Data availability statement

Data will be made available on request.

## Additional information

Supplementary content related to this article has been published online at [URL].

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.A.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.heliyon.2023.e14620>.

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