Science of the Total Environment 924 (2024) 171533

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Emerging nitrogen-fixing cyanobacteria for sustainable cotton cultivation

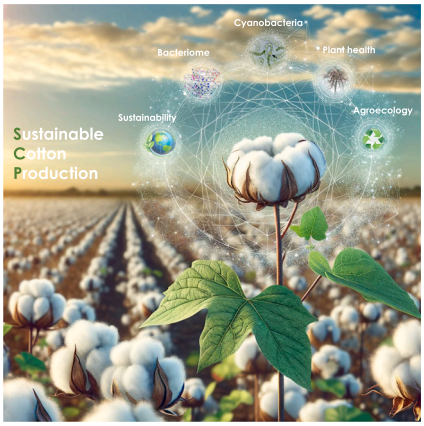
Lucía Jiménez-Ríos, Alejandro Torrado, José Luis González-Pimentel, Macarena Iniesta-Pallarés, Fernando P. Molina-Heredia, Vicente Mariscal *, Consolación Álvarez *

Instituto de Bioquímica Vegetal y Fotosíntesis, Consejo Superior de Investigaciones Científicas and Universidad de Sevilla, Américo Vespucio 49, 41092 Sevilla, Spain

HIGHLIGHTS

- Explored the dynamics of soil nutrient and bacterial diversity in cotton cultivation environments
- Employed metabarcoding techniques for in-depth analysis of soil bacterial communities associated with cotton fields
- Isolated and genetically characterized of native N_2-fixing cyanobacterial strains from cotton soil
- Uncovered plant growth-promoting activities in isolated cyanobacterial strains
- Demonstrated the efficacy of cyanobacteria as biofertilizers in enhancing cotton growth

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Abasiofiok Mark Ibekwe

Keywords:
Biofertilizers
cyanobacteria
Fertilizers
Metabarcoding
Nitrogen
Nitrogen pollution

ABSTRACT

Amid growing environmental concerns and the imperative for sustainable agricultural practices, this study examines the potential of nitrogen-fixing cyanobacteria as biofertilizers, particularly in cotton cultivation. The reliance on synthetic nitrogen fertilizers (SNFs), prevalent in modern agriculture, poses significant environmental challenges, including greenhouse gas emissions and water system contamination. This research aims to shift this paradigm by exploring the capacity of cyanobacteria as a natural and sustainable alternative. Utilizing advanced metabarcoding methods to analyze the 16S rRNA gene, we conducted a comprehensive assessment of soil bacterial communities within cotton fields. This study focused on evaluating the diversity, structure, taxonomic composition, and potential functional characteristics of these communities. Emphasis was placed on the isolation of native N_2-fixing cyanobacteria strains rom cotton soils, and their subsequent effects on cotton growth. Results from our study demonstrate significant plant growth-promoting (PGP) activities, measured as N_2 fixation, production of Phytohormones, Fe solubilization and biofertilization potential of five isolated cyanobacterial strains, underscoring their efficacy in cotton. These findings suggest a viable pathway for replacing chemical-synthetic nitrogen fertilizers with natural, organic alternatives. The reintegration of these beneficial species into agricultural ecosystems can enhance crop growth while fostering a balanced microbial environment, thus contributing to the broader goals of global sustainable agriculture.

* Corresponding authors.
E-mail addresses: vicente.mariscal@ibvf.csic.es (V. Mariscal), consolacion@ibvf.csic.es (C. Álvarez).

https://doi.org/10.1016/j.scitotenv.2024.171533
Received 15 January 2024; Received in revised form 4 March 2024; Accepted 4 March 2024
Available online 7 March 2024

1. Introduction

In the current era, marked by an increased awareness of environmental issues, there is a critical need for agriculture to shift towards more sustainable methods that reduce dependence on polluting agrochemicals. Prominent among these agrochemicals are synthetic nitrogen fertilizers (SNFs), whose manufacture and use result in significant environmental challenges (Zhang et al., 2015). A notable concern associated with the creation of SNFs is the substantial emission of greenhouse gases, particularly carbon dioxide and nitrous oxide, during the production process. This emission is a considerable contributor to global climate change and observed warming trends (Erisman et al., 2008; Galloway et al., 2008). Additionally, the widespread use of SNFs, a significant source of nitrogen compounds, can leach into both groundwater and surface water systems, leading to severe water contamination (Howarth and Marino, 2006). This results not only in eutrophication, but in the development of hypoxic conditions, a situation extensively reported in >150 coastal areas across Europe, especially at the junctions of major European river systems (Toral et al., 2011).

The continuous application of intensive farming methods, heavily driven by a steadfast dependence on synthetic agrochemicals, has steadily undermined soil health. Numerous studies have highlighted the harmful effects of intensive farming on the biodiversity of soil biomes and the intricate relationships among functional groups living in the soil (Tsiafouli et al., 2015; Huang et al., 2019; Wu et al., 2021; Iniesta-Pallarés et al., 2023). The organisms in these ecosystems form complex interdependent networks, playing crucial roles in processes such as nutrient cycling, decomposition of organic matter, natural pest control, and maintaining soil health. A reduction in soil biodiversity often leads to a decrease in the variety of functional groups of soil organisms, resulting in disturbances in these essential ecological activities. These disruptions have profound consequences for soil functionality and broader ecological processes (Iniesta-Pallarés et al., 2023).

A prominent example of this issue is evident in the ecologically rich Guadalquivir marshes, located within Doñana National Park in Southern Spain — an area renowned for its exceptional diversity of birdlife and its crucial position on the migratory routes used by various bird species between Europe and Africa (Fernández-Delgado, 2017). It's important to highlight that this area includes extensive cotton farms, covering roughly 38,000 ha mainly on the Guadalquivir River's left bank, representing more than half of Spain's cotton production. This contribution is significant within the European context, with only Greece having similar production levels. Cotton farming requires agronomic practices heavily dependent on synthetic fertilizers and pesticides, intensive soil plowing, and maintaining high soil moisture, particularly during the vegetative phase of plant when nitrogen is crucial (Zorrilla-Miras et al., 2014; Yang et al., 2011; Khan et al., 2017). This leads to a series of environmental problems, including soil fertility loss, increased water usage, and pollution (Cuevas et al., 2008; Singh et al., 2016).

Exploring the use of innovative organic or bio-based fertilizers that include plant growth-promoting rhizobacteria (PGPR) or bacteria (PGPB) presents a promising strategy. These microorganisms can improve soil texture, increase water retention and boost crop yields by supplying essential nutrients, vitamins, amino acids, and phytohormones (Singh et al., 2016). They are also integral in mitigating diseases and enhancing defense response against various pathogens, promoting a more robust agricultural system (Ngalimat et al., 2021). Utilizing PGP bacteria represents an eco-friendly method, reducing pollution and offering a cost-effective solution, thus supporting sustainable agriculture ideals. Among other sustainable agriculture options, nitrogen-fixing cyanobacteria have been highlighted recently as promising plant growth-promoting rhizobacteria (PGPR) (Iniesta-Pallarés et al., 2021; Zhang et al., 2021a, 2021b; Kumar et al., 2010; Singh et al., 2016).

These phototrophic microorganisms, capable of developing oxygenic photosynthesis, are prevalent in soil ecosystems, notably within the aquatic-terrestrial nexus of the Guadalquivir marshes. This natural environment is marked by favorable conditions that promote their growth (Iniesta-Pallarés et al., 2021). Interestingly, their ecological role extends beyond individual existence; a significant number engage in symbiotic relationships with a wide variety of plant life, covering an extensive phylogenetic spectrum within the plant kingdom (Adams and Duggan, 2012; Santi et al., 2013; Warshan et al., 2018; Álvarez et al., 2020; Álvarez et al., 2023).

A distinctive trait of nitrogen-fixing cyanobacteria resides in their faculty to facilitate the reduction of atmospheric nitrogen to ammonia, thereby offering a bioaccessible nitrogen reservoir to the plant (Kumar et al., 2010; Fugita and Uesaka, 2022). Nitrogen fixation occurs within specialized cells known as heterocysts, facilitated by the action of the nitrogenase enzyme complex (Kumar et al., 2010). This biochemical capability is especially crucial in nitrogen-deficient environments, allowing for biological nitrogen deposition in soils (Rousk, 2022). Rice farming has notably been a central focus of research regarding the use of cyanobacteria as biofertilizers, due to the warm and moist conditions of rice paddies that promote quick cyanobacterial growth (Prasanna et al., 2009; Priya et al., 2015; Iniesta-Pallarés et al., 2021). However, there is a significant knowledge gap concerning the effects of applying cyanobacteria in the cultivation of other important agricultural crops, like cotton. Furthermore, current studies mainly focus on using cyanobacterial extracts instead of live organisms, marking an area ripe for additional investigation (Gurusaravanan et al., 2013; Triveni et al., 2015).

In this study, we conducted an extensive analysis of bacterial communities in the soil of cotton fields. Using metabarcoding methods targeting the 16S rRNA gene, we uncovered the diversity, structure, taxonomic composition, potential functional characteristics, and assembly patterns of prokaryotic communities in bulk soils during a growing season. We further focused our analyses on a set of five native cyanobacterial strains, isolated from cotton soils. These strains were analyzed for their plant growth-promoting (PGP) activities and their effect on cotton growth, aiming to expand the existing understanding of cyanobacterial biofertilization capabilities in cotton.

Our results highlight the importance of biofertilization potential of the cyanobacterial strains we isolated, showing their effectiveness in boosting plant growth. This emphasizes the potential use of these strains as natural organic fertilizers, ready to supplant current chemical-synthetic nitrogen fertilizers. Additionally, reintroducing these beneficial strains to the ecosystem offers both, the benefit of enhancing cotton plant growth and nurturing a healthy microbial environment. This approach paves the way for sustainable agricultural practices based on beneficial microbial relationships.

2. Materials and methods

2.1. Site description and sample collection

The study area is situated in the Lower Guadalquivir region (southwest Spain). This area experiences a mild, temperate climate heavily influenced by its proximity to the Atlantic Ocean, which moderates temperatures and augments humidity levels. The annual average temperature and precipitation are 18 °C and 600 mm, respectively. Cotton has been cultivated in this region for over four decades, with fields annually receiving nitrogen fertilizers, typically 315 kg of urea per hectare, to boost production.

For the bulk soil bacteriome analysis, we selected an experimental plot located at Latitude 37°06′15.8“N and longitude 5°52’46.6”W. Soil samples were collected in 8 replicates at two separate intervals: one month after planting (vegetative phase, time 1) and two months after planting (reproductive phase, Time 2), yielding a total of 8 soil samples. Samples were transported to the laboratory and promptly stored at −80 °C. Throughout the study period, the experimental location was subject to the region's characteristic Mediterranean summer weather, marked by high temperatures that fluctuated between 16.1 °C and

42.9 °C, with an average of 30.6 °C. The absence of rainfall and predominantly clear skies provided optimal conditions for the cultivation of cotton. Accordingly, the crop received flood irrigation treatments twice within this timeframe.

2.2. Soil analysis

A fraction of each sample was air-dried and sieved to <2 mm for chemical analyses. Soil pH was measured in a 1:2.5 soil- KCl 1 M suspension after 30 min of shaking. Total Kjeldahl nitrogen (TKN) was analyzed in an automatic distillation system with titration, Vapodest 50S (Gerhardt). Total NO_3^-, NH_4^+ and P (Olsen) was determined in an automatic segmented flow analyzer Bran- Luebbe. X-ray fluorescence (XRF) spectroscopy (XRF Niton™) was used to measure total soil cations (Ca, K, Zn, Cu, Fe, Mg and Mn).

2.3. DNA extraction and Illumina sequencing

For the bacteriome analysis, DNA was extracted with NucleoSpin® Soil Kit following the instructions of the supplier. After quantification and analysis of DNA quality, they were subjected to Illumina amplicon sequencing at Eurofins Genomics Services (Ebersberg, Germany; https://eurofinsgenomics.eu/). The V4-V5 hypervariable regions of the 16S rRNA gene were sequenced to determine bacterial communities in all the 8 sample sets from bulk soils. The universal primers F515 (5'-GTGYCAGCMGCCGCGGTAA-3′) and R907 (5'-CCGTCAATTCMTTTRAGTTT-3′) (Zheng et al., 2018) were used in the sequencing process and a paired-end 2 × 300 bp strategy was followed.

The dataset obtained by the high-throughput sequencing is publicly available at the repository National Center for Biotechnology Information Sequence Read Archive (NCBI SRA) under the accession number PRJNA1054016.

2.4. Bioinformatic processing of sequencing data

Pre-processing and quality control filtering, operational taxonomic units (OTUs) picking, and taxonomy assignment were performed using Galaxy web platform at https://usegalaxy.org/ (Afgan et al., 2018). Theses analyses were developed using MOTHUR Project tools (Schloss, 2020; Schloss et al., 2009).

Bioinformatic analyses were carried out according to Iniesta-Pallarés et al. (2023). In brief, paired-end reads were merged, and sequences were trimmed according to the length of the amplified fragment. Sequences represented <10 times were discarded. Quality-control parameters as long homopolymer stretches and ambiguous base calls were selected, and chimeric sequences were removed using the VSEARCH algorithm (Rognes et al., 2016).

For the taxonomic classification version v138.1 of the SILVA database was selected (Quast et al., 2013), as a specific database for the analyses of 16S rRNA gene. After this first classification, non-bacterial sequences (chloroplasts, mitochondria, archaea, eukaryotes, and unknown sequences) were removed from the analysis.

2.5. Analysis of alpha diversity and prediction of functional profiles based of bacterial taxonomy

All statistical analyses were performed in R using base functions and the packages MicrobiomeAnalyst (Lu et al., 2023; Chong et al., 2020; Dhariwal et al., 2017) and *vegan* (Oksanen et al., 2022). The webservers of MicrobiomeAnalyst, www.microbiomeanalyst.ca, and MetagenAssist (Arndt et al., 2012), www.metagenassist.ca, were also used.

Firstly, we filtered the OTUs based on their prevalence. OTUs with <10 reads in <10 % of the samples were discarded. Then, the sampling effort was checked by calculating rarefaction curves for each sample (Rarefaction.single function for generating rarefaction curves, Galaxy Version 1.39.5.0). Finally, in order to remove potential biases related to different sequencing depth, samples were rarefied to 56,690 random reads per sample, the lowest library size of the whole dataset.

To estimate the alpha diversity (α-diversity) of the bacterial communities, ecological indices (observed OTU richness, Chao1 index, ACE index, Shannon diversity and evenness indices, Simpson and Fisher indices) were calculated. Diversity indices distributions were tested for normality and homogeneity of variances, and for not normal distribution Wilcoxon-Mann-Whitney (WMW) test was carried out. Permutational analysis of variance (PERMANOVA) was used to test the differences between sample groups using the *vegan:adonis* function (Oksanen et al., 2022).

In order to determine the functional traits of soil bacterial communities, the metabolic profile of bacterial communities was predicted using MetagenAssist webservice. To this end, the rarefied OTU table was used to predict bacteriome functions. The data was then normalized across samples and across variables by sum.

2.6. Isolation of cyanobacteria

Samples for cyanobacteria enrichment and isolation were obtained from same fields used for the bacteriome analysis (Supplementary Table S1). For the isolation of N_2-fixing cyanobacteria, samples containing 5 g of soil were suspended in 50 mL $BG11_0$ medium, lacking combined nitrogen (Rippka et al., 1979), and incubated at 30 °C overnight in continuous light (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Subsequently, a series of ten-fold dilutions were made and spread on agarose-solidified $BG11_0$ medium plates in the presence of cycloheximide (0.1 $mg \cdot mL^{-1}$) to inhibit microalgae and fungi growth. Plates were incubated for two weeks until the first cyanobacterial colonies appeared. 111 colonies were picked and successively re-streaked in $BG11_0$ medium plates in the presence of cycloheximide until individual colonies were obtained. Finally, colonies were grouped based on differences in color, growth and morphology. To isolate individual strains, a classical method of streaking on agar plates was used. Cyanobacterial strains were maintained in agar-solidified $BG11_0$ medium plates (Rippka et al., 1979).

2.7. DNA extraction and PCR amplification

Genomic DNA from cyanobacteria was extracted utilizing the NZY Tissue gDNA Isolation Kit (NZYTech) with some modifications. For the genetic characterization of the cyanobacterial isolates, a PCR-based approach was used (Iniesta-Pallarés et al., 2021). A conserved hypervariable genomic region presents ubiquitously in filamentous cyanobacteria served as the template for the synthesis of degenerate primers: FsepJ (5'-CAACAYGAHRTBYA-3′) and RsepJ (5'-CAYRTYCAAMCDTCHCT-3′). These primers were used to amplify this hypervariable genomic region of the isolated cyanobacterial strains by colony PCR with iProof™ High-Fidelity DNA Polymerase (Bio-Rad). PCR products were sequenced in Eurofins Genomics (Ebersberg, Germany).

2.8. Bioinformatic analyses and phylogenetic tree construction

All sequences from the different strains were subjected to a BLAST search against the GenBank nucleotide database to identify the closest matches (Table 3). For the bioinformatic evaluations, we aligned the sequences obtained along with other reference sequences from filamentous cyanobacteria sourced from GenBank, utilizing the MUSCLE algorithm set to a maximum of 8 iterations. The phylogenetic tree was formulated using the neighbour-joining (NJ) method and the Tamura–Nei genetic distance model, both facilitated by Geneious version 2020.0 developed by Biomatters (accessible at https://www.geneious.com, retrieved in 2021). Tree topology was assessed, and a bootstrap re-sampling was conducted, applying a 50 % support threshold and 10,000 replicates.

2.9. Light microscopy

For standard light microscopy, cyanobacterial strains were incubated in $BG11_0$ medium for 5 days at 25 °C in the light (50 μE·m^{-2}·s^{-1}) on a rotary shaker (100 rpm). Samples were taken with care and directly visualized by a Leica microscope with a 40× objective. For trichome length measurement, at least 100 trichomes were counted. Percentage of heterocysts was calculated from at least 1400 cells for each strain in >20 different fields. Dividing cells were counted as two cells.

2.10. In vitro screening plant growth promotion (PGP) activities

2.10.1. Determination of phytohormones

Selected strains, along with *Nostoc punctiforme* (as a model cyanobacterium control from the Institute of Plant Biochemistry and Photosynthesis), were cultured in 25 mL of $BG11_0$ liquid medium, enriched with 500 μg/mL tryptophan, at 25 °C under continuous illumination (50 μE·m^{-2}·s^{-1}). These shaking liquid cultures (100 rpm) were maintained for two weeks until they reached the stationary growth phase, with an initial inoculum density of 0.5 μg Chl·mL^{-1}. For analysis, 1 mL samples were centrifuged at 5000 ×*g* for 5 min. The resulting pellet was used for chlorophyll quantification, while the supernatant underwent LC-MS analysis for hormone concentration, normalized against the chlorophyll content in each cyanobacterial culture.

Hormone extraction from cyanobacterial samples (four biological replicates per strain) was performed using 1 mL of pre-cooled (20 °C) MTBE/MeOH solvent. Following vortex mixing, samples were agitated on an orbital shaker for 30 min at 4 °C, then sonicated for 15 min in an ice-cooled bath sonicator. After adding 0.5 mL of acidified water (0.1 % HCl) and vortexing for 1 min, samples were returned to the shaker for an additional 30 min at 4 °C. Post-centrifugation at 10,000 ×*g* for 10 min at 4 °C, 0.8 mL of the upper MTBE phase was transferred to a new tube and evaporated at 25 °C in a Speed-Vac concentrator, taking up to 1 h at 30 °C. Pellets were re-dissolved in 100 μL of a water:methanol (50:50) mixture for UPLC-ESI-MS/MS analysis, quantifying phytohormones including Salicylic acid (SA), Gibberellin A3 (GibA3), Indole-3-Carboxylic Acid (ICA), Indole-3-Acetic Acid (IAA), trans-Zeatin (tZ), Abscisic Acid (ABA) and Jasmonic Acid (JA).

Analysis was conducted using an Exion LC/Qtrap 6500+ (Sciex) with a Kinetex XB-C18 2.6 μM RP column (Phenomenex). A binary solvent system of water with 0.1 % (*v*/v) formic acid and acetonitrile with 0.1 % (v/v) formic acid was employed. Data acquisition and processing were facilitated through Analyst (version 1.7) and Sciex OS software (version 1.6.1), respectively.

2.10.2. Determination of nitrogenase activity

Nitrogenase activity was assessed using a modified Stewart et al. (1967) protocol. Cyanobacterial cultures were grown in $BG11_0$ medium to achieve a chlorophyll concentration > 20 μg/mL. Cultures were incubated with an initial inoculum density of 20 μg Chl·mL^{-1} at 25 °C under continuous light (50 μE m^{-2}·s^{-1}) and stirred at 100 rpm. Each strain was replicated three times. To inhibit gas exchange, flasks were sealed with rubber caps. After 30 min of incubation, 2 mL of acetylene was injected into each flask, followed by 1 mL gas samples taken every 30 min for 2.5 h. Ethylene production was measured using gas chromatography, with nitrogenase activity reported in μmol ehtylene·g^{-1} Chl·s^{-1}.

2.10.3. Assessment of siderophore production

Siderophore production was evaluated both qualitatively and quantitatively using the competitive iron assimilation method on chrome azurol S/Fe (III)/hexadecyltrimethyl-ammonium bromide (CAS), as described by Pérez-Miranda et al. (2007). For the qualitative method, cyanobacterial strains were grown on solid agar $BG11_0$ medium at a constant 25 °C for 7 days and then CAS agar solution was placed on top. The formation of a yellow or white halo, displacing the initial blue color of the media around the colony, was considered a positive indication of siderophore production.

Quantitative estimation of siderophore production in liquid cultures was adapted from Schwyn and Neilands (1987). Pre-inoculum of cyanobacterial strains in exponential phase was washed and inoculated in the absence Fe for 4 days under continuous light (50 μE m^{-2} s^{-1}), at 25 °C. Then, the extracellular medium was mixed 1:1 (v/v) CAS assay solution. As reference was prepared using the uninoculated culture media. The mix was incubated for 1 h at RT and absorbance was determined at 630 nm. Three replicates were made. Siderophores produced by strains was measured in percent siderophore unit (psu) which was calculated according to the following formula (adapted from Payne, 1993):

$$\text{Siderophore production (psu)} = \frac{\left(\frac{(Ar-As)}{Ar}\right)}{Chl} x100.$$

where A_r = absorbance of reference (CAS solution and uninoculated culture), A_s = absorbance of sample (CAS solution and cell-free supernatant of sample) and Chl = concentration of chlorophyll (μg Chl·mL^{-1}).

2.11. Cotton seed planting and cyanobacterial inoculation

Cotton seeds were sown in 1-l pots filled with soil, in a greenhouse set at 25 °C and a 14/8 h day/night cycle. Five native cyanobacterial isolates (G23, G26, G35, G40, G42) and *N. punctiforme* PCC 73102 were used for inoculation. Each strain was initially cultured in 100 mL sterile $BG11_0$ medium in 250 mL flasks, incubated for 14 days at 25 °C with continuous light (50 μE m^{-2}·s^{-1}) and shaking (100 rpm). Cultures were then expanded to 700 mL in Roux bottles with 10 mM $NaHCO_3$ and 1 % CO_2, incubated for 7 more days at 30 °C under the same light conditions. Biomass was harvested by centrifugation at 4000 ×*g* for 10 min at room temperature and diluted to 1.5 μg Chl·mL^{-1}.

Each treatment was replicated thrice, with twelve pots per replication. Treatments involved two inoculations of 10 mL of each strain (1.5 μg·mL^{-1}) per pot at two stages: initial sowing and 14 days post-planting. The plants were grown in a greenhouse under controlled temperature for 30 days. The control treatment used sterile $BG11_0$ medium without cyanobacteria.

Post 30 days of growth, initial assessments of leaf and root lengths were conducted. Plants were then uprooted for biomass analysis, separating and drying aerial and root sections at 70 °C for 3 days to measure dry weight.

3. Results

3.1. Soil physicochemical analyses

The analysis conducted to assess the impact of fertilization and soil condition changes during cultivation (one month and two months after planting) highlighted specific nutrient variations. Notably, nitrate (NO_3^-) levels significantly declined, from 121.17 ± 43.99 mg/kg to 50.33 ± 14.18 mg/kg. Meanwhile, manganese (Mn) levels demonstrated a significant increase, from 43.80 ± 0.83 mg/kg to 52.66 ± 3.09 mg/kg. In contrast, other macro- and micronutrient levels remained relatively stable throughout the cultivation period (Table 1).

3.2. Bacteriome analysis from cotton soils

To explore bacterial richness in soils used for cotton cultivation and the effects of fertilization and agricultural practices, a detailed bacteriome analysis was performed using high-throughput amplicon sequencing. From 16 soil samples, we obtained 1,437,454 raw sequences, with an average of 89,840 reads per sample. Following stringent quality filtering, which included the removal of singletons,

Table 1
Soil parameters at the different stages of cotton cultivation. T1, 1 month after planting; T2, two months after planting. Mean and standard error are shown. Phosphate (P), Potassium (K), Nitrite (NO_3^-), Magnesium (Mg), Calcium (Ca), Iron (Fe), Manganese (Mn), Zinc (Zn) and Copper (Cu).

	T1	T2
pH	7.96 ± 0.07	8.10 ± 0.05
Salinity (mS/cm)	0.58 ± 0.09	0.39 ± 0.08
Organic C (%)	1.07 ± 0.04	1.09 ± 0.10
Organic Matter (%)	1.85 ± 0.07	1.88 ± 0.18
N Kjeldahl (%)	0.10 ± 0.01	0.10 ± 0.01
NO_3^- (mg/kg)	121.17 ± 43.99	50.33 ± 14.18
NH_4^+ (mg/kg)	3.83 ± 0.53	2.20 ± 0.32
P (mg/kg)	13.63 ± 1.38	12.70 ± 0.70
K (g/kg)	1.04 ± 0.20	1.16 ± 0.13
Ca (g/kg)	6.33 ± 0.30	6.98 ± 0.08
Mg (g/kg)	0.74 ± 0.07	0.68 ± 0.03
Fe (mg/kg)	22.01 ± 3.30	20.93 ± 0.77
Cu (mg/kg)	5.71 ± 0.32	5.28 ± 0.28
Mn (mg/kg)	43.80 ± 0.83	52.66 ± 3.09
Zn (mg/kg)	1.37 ± 0.09	1.06 ± 0.07

chimeric, low abundance, and non-bacterial sequences, a total of 1,326,097 high-quality sequences were retained. Subsequently, a rarefaction process was applied, standardizing the data to a minimum of 56,690 random reads per sample. These sequences were further clustered into 3789 operational taxonomic units (OTUs) at 97 % genetic identity (Supplementary Table 1).

Alpha diversity indices, such as Shannon and Simpson, showcased high bacterial diversity in all samples, indicating a rich microbial environment (Table 2). Principal Coordinates Analysis (PCoA) using weighted UniFrac distances, along with PERMANOVA test results ($F = 3.0868$; $p = 0.001$), suggested no significant differences in microbial distribution or temporal variation (see Supplementary Fig. 1). Consequently, all samples were combined for further analysis. Taxonomic examination revealed dominant bacterial phyla including *Pseudomonadota* and *Actinomycetota*, as the most abundant groups in both Time 1 and Time 2, with a noticeable representativeness of *Acidobacteriota*, *Chloroflexota*, *Gemmatimonadota*, *Bacillota*, *Bacteroidota*, *Planctomycetota*, *Myxococcota* and *Candidatus* Tectomicrobia, all of them exceeding the 1 %, depicting a varied bacterial community in the cotton soil ecosystem (illustrated in Fig. 1A). Remarkably, up to 29 bacterial groups at family level presented an abundance above the 1 %, highlighting *Gemmatimonadaceae*, uncultured *Gaiellales*, *Azospirillaceae* and *Geminococcaceae*, all of them ranging the 3–6 % of the total abundance. Regarding to the representativeness of identified taxons, it is noticeable the genus *Skermanella* (3.27 ± 1.24 %), afilliated to the phylum *Pseudomonadota*, followed by *Geodermatophilaceae* (2.20 ± 0.53 %) and uncultured *Gaiellales* (1.89 ± 0.63 %), grouped in the phylum *Actinomycetota* (detailed in Supplementary Table 1). These results showed a notable heterogeneity in the microbial community of the analyzed soils, with none of the identified taxa being significantly overrepresented and many groups with less than the 1 % of the relative abundance. Among them, beneficial N_2-fixing bacteria such as the phylum *Cyanobacteria*, and more concretely the family *Nostocaceae*, reaching the 0.09 % and 0.04 %, respectively, as well as the family *Rhizobiaceae*, grouped in the *Rhizobiales* order (exceeding the 4 %), representing up to the 0.76 %.

The metabolic potential of the soil bacteria was assessed using their taxonomic profiles processed through METAGENassist, providing information for 45 % of the bacterial community (Arndt et al., 2012). Analysis of oxygen requirements revealed the presence of both anaerobic (12.02 ± 1.92 %) and aerobic (87.97 ± 1.92 %) bacteria. Among the 106 primary metabolic activities in the METAGENassist database, key functions related to nitrogen (N) cycle, sulfur (S) cycle, and pollutant degradation were identified (illustrated in Fig. 1b). For the N cycle, notable functions included ammonia oxidation (14.02 ± 0.38 %), nitrite reduction (10.04 ± 0.39 %), and nitrogen fixation (4.08 ± 0.45 %). In the S cycle, sulfate reduction (10.44 ± 0.43 %) and sulfide oxidation (10.34 ± 0.47 %) were prominent. Additionally, a significant array of degradative metabolisms related to agrochemical degradation, such as dehalogenation (9.61 ± 0.44 %), was observed.

Table 2
Alpha diversity indices. Mean values and standard error of the mean is indicated for each group of samples. T1 (one month after planting) and T2 (two months after planting). Obs = Number of observed OTUs.

Sample	Obs	Chao1	ACE	Shannon	Simpson	Fisher
T1	696.87 ± 83.95	704.80 ± 86.98	701.62 ± 85.85	5.56 ± 0.09	0.99 ± 0.0009	114.58 ± 15.90
T2	813.37 ± 45.69	824.63 ± 48.24	819.90 ± 47.29	5.61 ± 0.04	0.99 ± 0.0005	136.52 ± 9.08

3.3. Isolation and genetic characterization of nitrogen-fixing cyanobacteria from cotton soils

In order to provide a sustainable alternative to the use of nitrogen fertilizers for cotton cultivation, we addressed the isolation of nitrogen-fixing cyanobacteria, as potential candidates for biofertilizers. Isolating these strains from soil samples, we aimed to investigate their PGP activities for potential soil restoration and plant health enhancement.

To characterize the isolated cyanobacteria, 26 strains were selected for genetic analysis from the 111 isolated colonies, based on colony morphology, color, and shape. A PCR approach targeting a genomic region specific to filamentous cyanobacteria was employed (Iniesta-Pallarés et al., 2021). After a post-amplification step and sequencing, the strains were categorized into five genotypes (Fig. 2). Phylogenetic analysis, using reference sequences from GenBank, revealed that strains G23 and G26 are closely related to *Nostoc* sp. within the same phylogenetic group. In contrast, strains G35, G40, and G42 each belong to different major phylogenetic groups, highlighting their genetic diversity.

3.4. Morphological description of the cyanobacterial isolates

A detailed morphological characterization of the five cyanobacterial strains was performed, analyzing cell dimensions, shape, color, trichome structure, and cell differentiation (Fig. 3), following the guidelines of Komárek (2016) and Rippka et al. (1979). For this analysis, cultures of each strain in BG11o were prepared. Despite most of the cultures grew in clumps, we did not observe any attachment to the wall of the Erlenmeyer flasks (see Supplementary Fig. 2). This analysis was complemented with a genetic examination of their 16S rRNA to support previous phylogenetic findings (Table 3).

G23 and G26 strains showed a close phylogenetic relationship with *Nostoc* sp. NIES-2111, in contrast to G35, G40, and G42, which were identified with separate key phylogenetic groups—*Anabaenopsis*, *Calothrix*, and *Anabaena*, accordingly. The strains G23 and G26 exhibited the highest number of cells per trichome, with averages of 43 ± 4.1 and 62.6 ± 6.2 cells, respectively. Trichomes of these strains were mildly curved. In contrast, the strains G40 and G42 exhibited a lower number of cells per trichome (19.2 ± 2.5 and 20.4 ± 1.2), both were characterized by conical terminal cells. The G35, G40 and G42 strains showed the highest percentage of heterocysts per vegetative cell, specifically 10 ± 0.6, 8.3 ± 1.3 and 8.1 ± 0.6, respectively. The presence of both akinetes and hormogonia was noted in all strains (Fig. 3 and Table 3).

3.5. Effect of cyanobacterial strains inoculation on plant growth under greenhouse conditions

We conducted a comprehensive evaluation of the plant growth-promoting effects exhibited by five cyanobacterial strains, with *Nostoc*

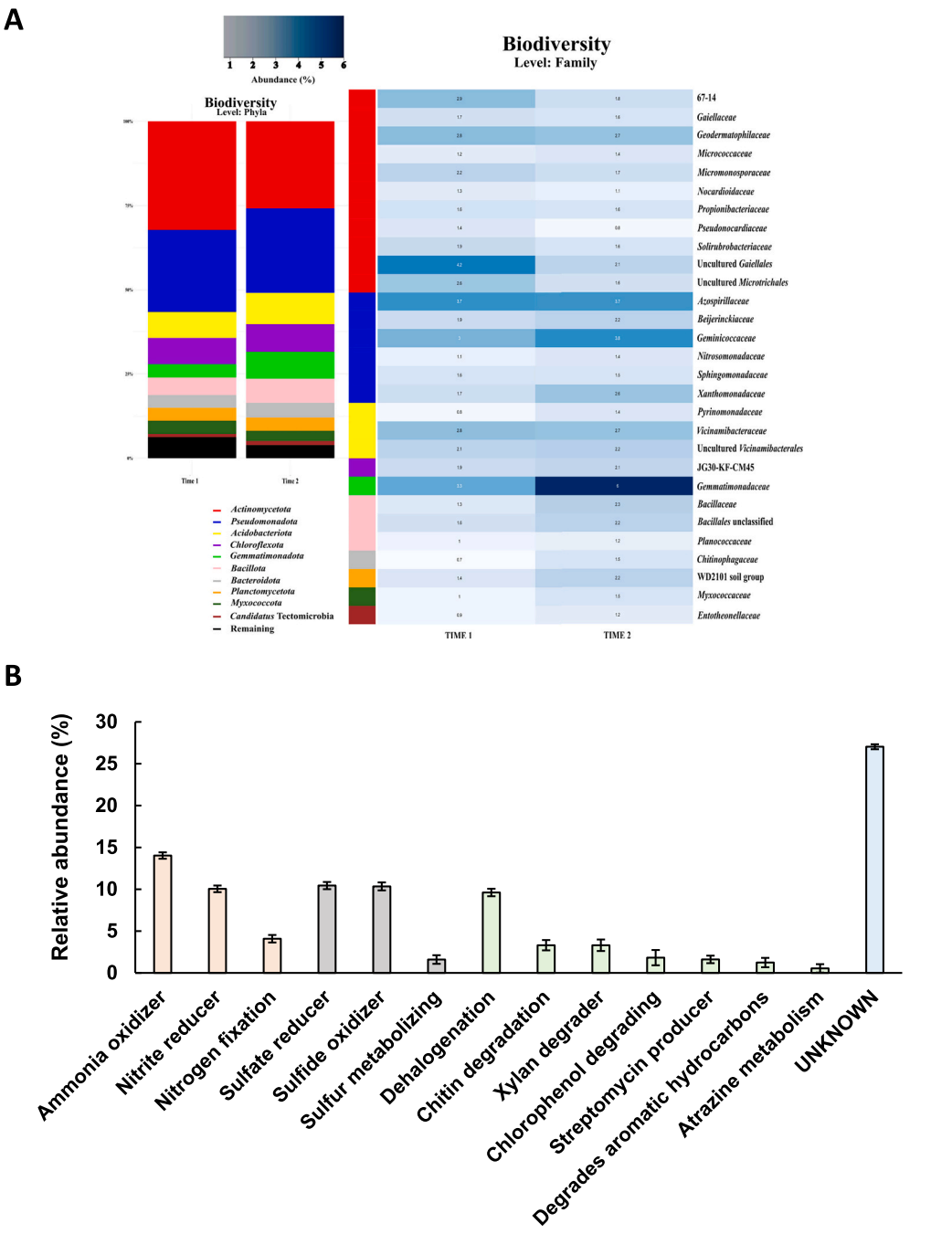


Fig. 1. Bacterial diversity and potential metabolic functions from cotton soils. A) Bar plot of the main phyla found in bulk soil samples at two times using the rarefied OTU table at a depth of 56,690 sequences per sample. Time 1 (one month after planting) and time 2 (two months after planting). B) Prediction of the metabolic profiles of the bacteriome from bulk soil samples using the METAGENassist web service database.

punctiforme serving as a comparative control. The assessment of biofertilization capabilities was meticulously performed through a combination of visual inspections, photographic documentation, and precise biometric analyses, including the measurement of aerial and root lengths, as well as the determination of dry weight (refer to Fig. 4 for visual representation).

Among the tested strains, G23 and G42 emerged as particularly potent in enhancing several key biometric parameters of the plants. Specifically, strain G23 was observed to significantly increased the size and weight of the plant's aerial parts. Compared to the untreated, control group, plants treated with G23 exhibited a remarkable 41 % increase in aerial size ($p = 0.001$) and a 56 % increase in aerial weight ($p = 0.012$), as depicted in Fig. 4A and B.

Similarly, the application of strain G42 resulted in a notable improvement in plant biomass, with an almost 50 % increase in size ($p = 0.003$) relative to the control, as illustrated in Fig. 4A and B. The impact of G42 was not confined to aerial enhancement; it also significantly promoted root growth. Specifically, plants treated with G42 exhibited a 48 % increase in root size ($p = 4.5{\cdot}10^{-6}$) and a notable 80 % increase in root weight ($p = 6.7{\cdot}10^{-5}$), compared to the control (Fig. 4A and C), significantly boosting the growth and development of lateral and adventitious roots.

While strains G26, G35, and G40 did not show statistically significant differences in the size or weight of the aerial or root parts when compared to the control, it is noteworthy that G35 and G40 contributed to a non-negligible improvement in the weight of the root parts by 27 %

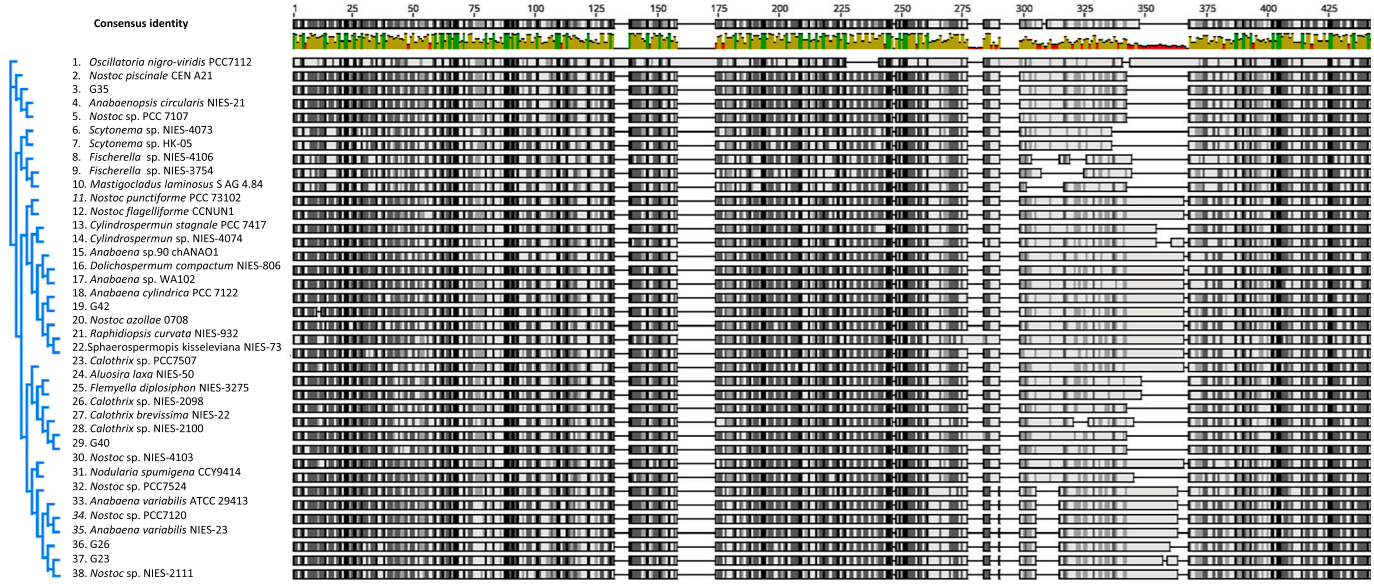


Fig. 2. Alignment of the sequences used for the genetic characterization of the cyanobacterial isolates. The genomic region contained a hypervariable region that was used to genetically trace the different cyanobacterial strains. Sequences were aligned by MUSCLE algorithm and the phylogenetic tree was formulated using the neighbour-joining (NJ) method. Figure was constructed with Geneious version 2020.0.

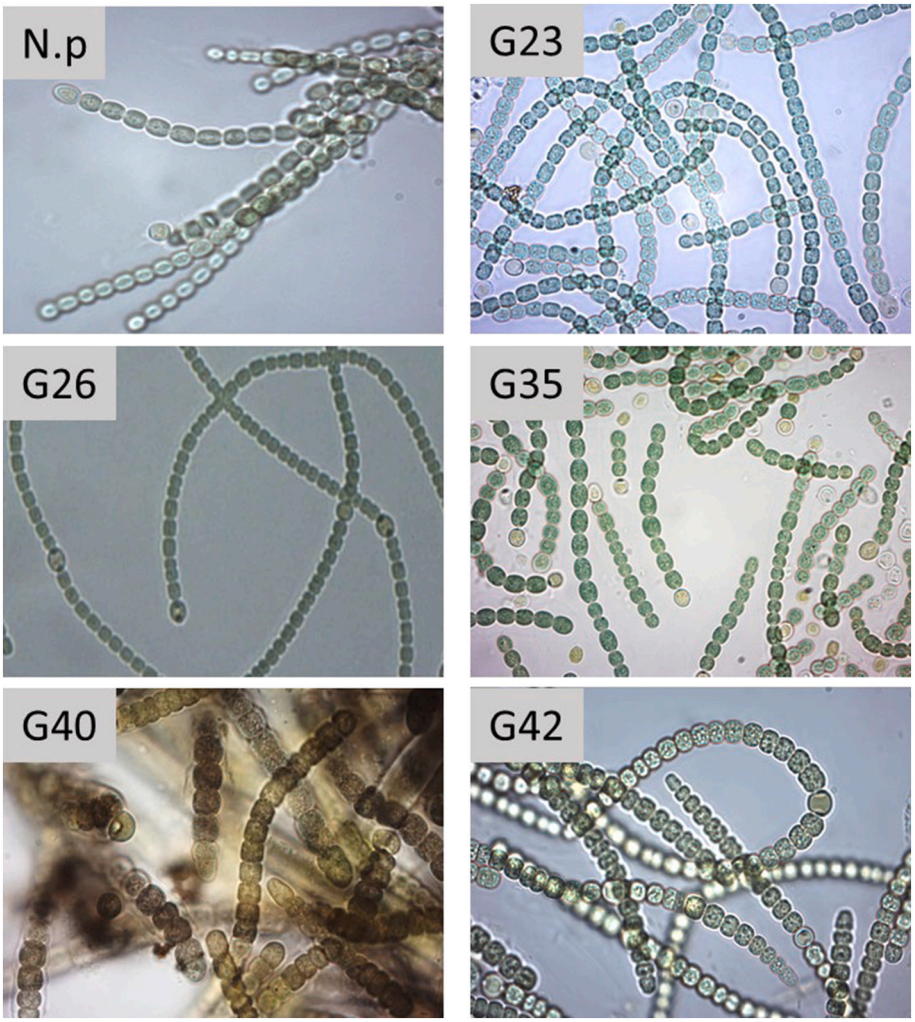


Fig. 3. Light micrographs of the cyanobacteria isolated from cotton soils. Liquid cultures of *Nostoc punctiforme* (N.p), G23, G26, G35, G40 and G42 strains were prepared in BG110 (without nitrogen) to induce the development of heterocysts. All micrographs were taken in a light microscope through a 40× objective.

Table 3
Morphological characters of cyanobacterial strains used in this study.

Strain	Culture color	Cells per trichome	Heterocysts (%)	Akinetes	Hormogonia	Closest related
G23	Green	43.0 ± 4.1	5.1 ± 0.4	Yes	Yes	*Nostoc* NIES 211
G26	Green	62.6 ± 6.2	4.1 ± 0.4	Yes	Yes	*Nostoc* NIES 211
G35	Blue-Green	24.9 ± 2.1	10.0 ± 0.6	Yes	Yes	*Anabaenopsis circularis* NIES-21
G40	Brown	19.2 ± 2.5	8.3 ± 1.3	Yes	Yes	*Calothrix* NIES 2100
G42	Green	20.4 ± 1.2	8.1 ± 0.6	Yes	Yes	*Anabaena cylindrica*

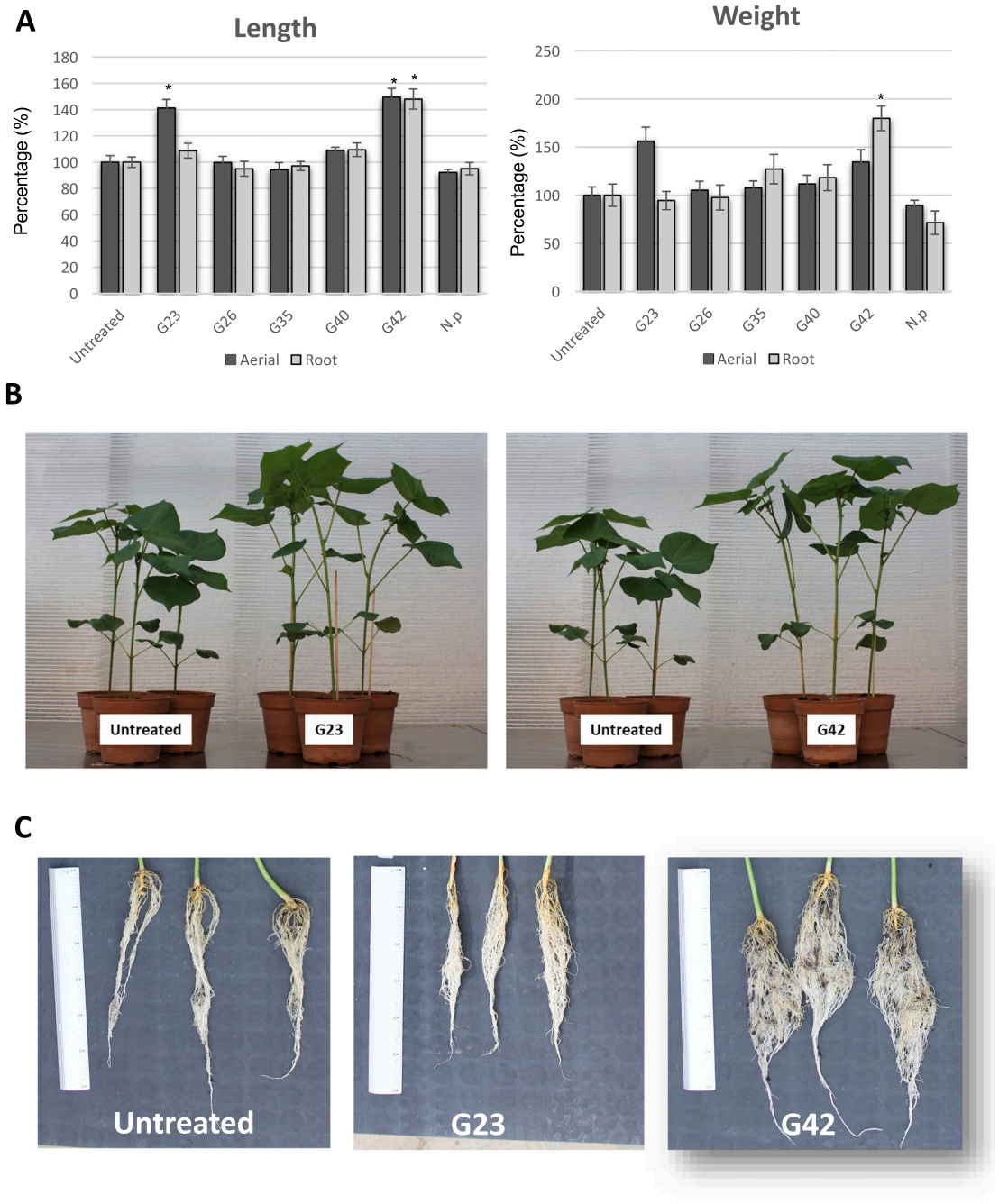


Fig. 4. Cotton growth enhancement by cyanobacterial treatment. A) Analysis of aerial and root growth in cotton plants measured by length and biomass 30 days after treatment with cyanobacterial strains: *Nostoc punctiforme* (N.p), G23, G26, G35, G40, and G42, compared with untreated controls. Values represent the mean ± standard error from three independent trials, with twelve replicates each. Statistically significant increases in growth ($p < 0.01$) are marked with an asterisk (*) according to Student's *t*-test. (B) Photograph of the aerial part and (C) roots of cotton plants cultivated in soil and treated with strains G23 or G42 at 30 days post-inoculation.

($p = 0.22$) and 18 % ($p = 0.36$), respectively (Fig. 4A). Although these increases did not reach statistical significance, they hint at a potential beneficial effect on root biomass, warranting further investigation into the conditions under which these strains may express more pronounced biofertilization effects.

3.6. Plant growth promotion activities

To further investigate the potential of cyanobacterial strains as plant biofertilizers, we analyzed three PGP activities: phytohormone production, nitrogenase activity, and siderophore production.

In our study of phytohormones, GibA3 and cytokinin tZ (trans-zeatin) production were observed in all strains, with G35 and *N. punctiforme* emerging as the predominant producers of GibA3 (19.89 ± 1.7 and 14.26 ± 1.2 μg·mg^{-1} Chl, respectively) and cytokinin tZ (0.27 ± 0.06 and 0.36 ± 0.04 μg·mg^{-1} Chl, respectively) (Table 4). Only *N. punctiforme*, G23, and G35 produced the auxin IAA, with *N. punctiforme* exhibiting the highest production level (1033.87 ± 72.0 μg·mg^{-1} Chl). Additionally, SA secretion was exclusive to G23 and G35, with G35 being the highest producer (44.48 ± 6.2 μg·mg^{-1} Chl) (Table 4).

Nitrogen fixation activity, a crucial PGP trait, was measured across all cyanobacterial strains, evaluated by ethylene production from acetylene. As expected from filamentous heterocyst-forming cyanobacteria, all strains demonstrated high nitrogenase activity. Notably, strains G23 and G42 exhibited the most pronounced activity levels, with 1.00 ± 0.06 and 1.06 ± 0.02 μmol ehtylene·g^{-1} Chl·s^{-1} respectively, which is about threefold higher than that of the model strain *N. punctiforme* (0.34 ± 0.05 μmol ethylene·g^{-1} Chl·s^{-1}; Table 4).

Siderophores, known for their iron-chelating ability and low molecular weight, offer significant benefits. They assist cyanobacteria in iron sequestration, essential for their metabolism, and enhance iron bioavailability in the rhizosphere, aiding plant uptake. All cyanobacterial strains demonstrated siderophore production on solid media, underscoring their potential role in iron acquisition and management in soil-plant systems. In particular, according to quantitative analysis, strains G23, G35, and G40 produced approximately twice the amount of siderophores compared to *N. punctiforme* (see Table 4 and Supplementary Fig. 3).

4. Discussion

Cotton production faces significant challenges, such as excessive fertilizer application, leading to environmental issues like eutrophication and greenhouse gas emissions (Zhang et al., 2021a). Understanding the richness of bacteria in soils and their composition is crucial for efficient fertilizer use in order to mitigate these problems (Jacoby et al., 2017; Zheng et al., 2021). This study provides an extensive analysis of cotton soils and offers a biological alternative to the use of chemicals, optimizing nutrient utilization in cotton plants.

We found high levels of nitrate in the soil, indicative of rapid transformation of nitrogen fertilizers by soil bacteria (Coskun et al., 2017; Grzyb et al., 2021; Klimasmith and Kent, 2022). The observed reduction in nitrate levels in our study, when the plant is producing the flower buds, is consistent with findings that highlight the complex interplay between nitrogen uptake by plants and leaching processes (Govindasamy et al., 2023). Such dynamics are critical in cotton cultivation, where nitrogen use efficiency (NUE) is a pivotal factor influencing both yield and environmental impact (Govindasamy et al., 2023; Chattha et al., 2022). However, over-application of nitrogen, often used to boost cotton growth, can paradoxically lead to reduced lint yield and NUE, underscoring the need for well-defined nitrogen management practices (Chattha et al., 2022). The increase in manganese levels might be linked to changes in soil pH or organic matter content, impacting the bioavailability of micronutrients and reflecting the complexity of soil nutrient dynamics during cotton cultivation, that might be related with the microbial diversity in the soil (Khoshru et al., 2023).

Our analysis of the soil bacteriome aligns with recent studies demonstrating the significant role of the soil bacteriome in modeling agricultural production (Adomako et al., 2022; Gupta et al., 2022; Nadarajah and Abdul Rahman, 2023). The high bacterial diversity observed in our samples, especially the presence of phyla such as *Pseudomonadota* and *Actinomycetota*, indicates a robust microbial ecosystem that is capable of enhancing soil quality and function by providing N, P and S transformations, as well as phytochemicals detoxification and disease suppressiveness. Identified taxa such as *Nitrosomonadaceae*, *Gaiellaceae*, *Micrococcaceae*, JG30-KF-CM45, *Xanthomonadaceae*, *Bacillaceae*, *Chitinophagaceae* and *Gemmatimonadaceae* are shown to be involved in ammonium and nitrite metabolisms (Prosser et al., 2014; Aanderud et al., 2018; Lu et al., 2020; Yang et al., 2021; Petrilli et al., 2023; Liu et al., 2024). Some of these bacteria would be associated with phosphorus cycle as well as C fixation and C degradation, such as *Vicinamibacteraceae*, *Pyrinomonadaceae*, 67–14 group, WD2101 soil group, *Gaiellaceae*, *Gemmatimonadaceae* and *Chitinophagaceae*, depicting the relevance of a heterogeneous community of bacteria in agricultural soils (Pérez-Castro et al., 2019; Shi et al., 2020; Lu et al., 2020; Company et al., 2022; Wang et al., 2022; Yang et al., 2023). In fact, distinct groups identified in this study, such as *Geodermatophilaceae*, *Micromonosporaceae*, *Propionibacteriaceae*, *Nocardiodaceae*, *Geminicoccaceae*, *Myxococcaceae*, *Entotheonellaceaehave* have been showed to display determinant roles in different processes, including plant-growth promotion, disease suppressiveness and secondary metabolites production, demonstrating the importance of a diverse bacterial community for healthy soils in agriculture (Xiao et al., 2017; Costa et al., 2023; Gu et al., 2023; Ma et al., 2023; Peters et al., 2023; Triviño et al., 2023). Likewise, nitrogen-fixing bacteria within the phyla *Pseudomonadota*, such as the family *Azospirillaceae* are essential for converting atmospheric nitrogen into a form that plants can use, thus enhancing soil fertility. The presence of these bacteria appears to be associated with other groups, as observed in a recent study where the *Azospirillaceae*-affiliated genus *Skermanella*, identified as the most abundant OTU in this analysis, which was correlated with the family members *Solirubrobacteriaceae* and *Gaiellaceae*, suggesting a direct implication in nitrogen metabolism processes (Chen et al., 2023). It is worth highlighting the presence of N_2-fixing bacteria with lower representation, such as cyanobacteria and groups affiliated to the order *Rhizobiales*, also found in nearby paddy soil

Table 4
PGP activities of the cyanobacterial strains. *N. punctiforme* (N.p). SA: Salicylic acid, GibA3: Gibberellin A3, ICA: Indole-3-Carboxylic Acid, IAA: Indole-3-Acetic Acid, tZ: trans-Zeatin. Siderophore production was measured in percent siderophore unit (psu) normalized to chlorophyll concentration.

	Phytohormones (μg/mg Chl)					Nitrogenase activity (μmol ethylene·g^{-1} Chl·s^{-1})	Siderophore production (psu)
Strain	SA	GibA3	ICA	IAA	tZ		
N.p	–	14.26 ± 1.2	1033.87 ± 72.0	7043.21 ± 497.6	0.36 ± 0.04	0.34 ± 0.05	2.04 ± 0.40
G23	1.26 ± 0.3	5.46 ± 0.4	370.51 ± 126.5	3244.41 ± 87.5	0.08 ± 0.02	1.00 ± 0.06	4.18 ± 0.46
G26	–	6.92 ± 0.6	–	4.95 ± 0.32	0.03 ± 0.003	0.56 ± 0.06	3.02 ± 0.21
G35	44.48 ± 6.2	19.89 ± 1.7	187.01 ± 32.4	7998.98 ± 534.3	0.27 ± 0.06	0.50 ± 0.03	4.89 ± 0.12
G40	–	5.81 ± 1.3	–	–	0.01 ± 0.002	0.19 ± 0.04	4.26 ± 0.14
G42	–	7.71 ± 0.9	–	–	0.02 ± 0.002	1.06 ± 0.02	2.27 ± 0.15

(Iniesta-Pallarés et al., 2023). Despite cyanobacteria not being the most represented family among nitrogen-fixers in this analysis, contribute significantly to nitrogen cycling. Cyanobacteria possess unique genetic and physiological traits that enable them to thrive in diverse environments, ranging from arid soils to aquatic habitats, often colonizing spaces where other nitrogen-fixers cannot (Adams and Duggan, 2012; Canfield et al., 2010; Hsu and Buckley, 2009). These findings highlight the importance of considering the composition and function of the bacteriome in sustainable agricultural management and in the improvement strategies for cotton crops.

The combination of both culture-dependent and culture-independent techniques has showed a useful approach for a deeper knowledge of the bacteriome (Wei et al., 2021; Sondo et al., 2023), since both methods separately present biases distorting the snapshot of the bacterial community (Al-Awadhi et al., 2013; Sidstedt et al., 2020). Furthermore, culture-dependent techniques allow the isolation of rare bacteria found in the bacteriome analysis, that might be important for plant growth (Krstić Tomić et al., 2023). Thus, our study embarked on a comprehensive investigation into the use of soil cyanobacterial strains as biofertilizers for cotton cultivation. This initiative was driven by the urgent need to develop alternative biofertilization strategies, especially in the face of increasing environmental challenges associated with the intensive use of SNFs. The application of cyanobacteria as biofertilizers is not a novel concept in agriculture, as these organisms have been extensively explored in other crop systems. For instance, notable advancements have been made in rice cultivation, where cyanobacteria contribute significantly to nitrogen fixation, thus enhancing soil fertility and crop yield (Prasanna et al., 2009; Priya et al., 2015; Zayadan et al., 2014; Iniesta-Pallarés et al., 2021; Song et al., 2021; Song et al., 2022). Furthermore, their role in the soil microbiome, providing essential nutrients has been well-documented (Singh et al., 2016; Iniesta-Pallarés et al., 2023). However, despite these successes in other crops, the potential of cyanobacteria in cotton cultivation has remained largely unexplored until our current study. Our findings reveal that these prokaryotic microorganisms exhibit potential for biofertilization, highlighting their beneficial role in promoting sustainable agricultural practices and aiding environmental conservation efforts. The positive implications observed in rice and other crops underline the versatility of cyanobacteria as biofertilizers, suggesting their broad applicability across various agricultural contexts. This study, therefore, contributes to the expanding body of knowledge on cyanobacterial biofertilization in cotton.

In our methodology, we employed isolation and characterization protocols that mirror those used for isolating N_2-fixing cyanobacteria from rice paddies (Iniesta-Pallarés et al., 2021; Vijayan and Ray, 2015). This approach highlights the versatility and effectiveness of these techniques in various agricultural contexts. Our objective was a predominant enrichment of cyanobacteria, which appeared to be notably less abundant in the soils studied through bacteriome analysis, alluding to the effectiveness of selective enrichment after using specific media for the isolation of cyanobacteria. The implications of our research extend beyond cotton cultivation, suggesting broader applications in sustainable agriculture and offering a promising avenue for reducing reliance on synthetic fertilizers.

Our phylogenetic analysis corroborated the categorization of the five heterocyst-forming nitrogen-fixing cyanobacteria studied. Strains G23 and G26 clustered closely with the *Nostoc* genus, whereas strains G35, G40, and G42 aligned with distinct major phylogenetic groups—specifically *Anabaenopsis*, *Calothrix*, and *Anabaena*. Notably, *Calothrix* sp., closely related to G40 is known for its ubiquitous presence in cultivated soils, and has been previously demonstrated to facilitate the growth of rice crops, highlighting its agricultural significance (Priya et al., 2015). Furthermore, *Anabaena cylindrica* PCC 7122, a strain of cyanobacterium closely related to G42, has been assessed for its efficacy as a biofertilizer in lettuce cultivations (Xue et al., 2017), suggesting the potential utility of these microorganisms in diverse agricultural crops. Our analysis of a hypervariable region, specific to filamentous cyanobacteria, led us to conclude that the strains we investigated are new and have not been previously unidentified. This genetic diversity was reflected in the morphological heterogeneity observed among the strains, consistent with the findings of Kumar et al. (2010), Fugita and Uesaka (2022), Roque et al. (2023) and Iniesta-Pallarés et al. (2021), who reported a broad range of morphological and genetic diversity among soil-inhabiting cyanobacteria.

The morphological and genetic diversity of soil cyanobacteria reveals novel cyanobacterial strains with unique properties that might be used to develop customized biofertilizers, specifically designed to meet the needs of different crops and varying environmental conditions. This approach is in line with the principles of sustainable agriculture. In our research, we delved into various PGP activities exhibited by these newly identified cyanobacterial strains and their impact on the growth of cotton plants. The primary objective was to broaden our understanding of how cyanobacteria could be effectively utilized as biofertilizers in cotton cultivation. Existing research indicates that cyanobacteria are capable of synthesizing a range of compounds that possess PGP properties. For instance, various strains of *Nostoc* and other filamentous cyanobacteria have been demonstrated to enhance crop growth, possibly through the biosynthesis of phytohormones, including auxins, cytokinins, salicylic acid, ABA, JA and giberellins (Sergeeva et al., 2002; Amin et al., 2009; Tan et al., 2021; Toribio et al., 2020, 2021; Bayona-Morcillo et al., 2020; Uniyal et al., 2022).

Our study examined five new cyanobacterial strains, discovering that only G23 and G35 produced salicylic acid, a phytohormone critical for plant defense and physiological processes (Toribio et al., 2020). Additionally, salicylic acid plays a role in modulating several plant physiological processes, encompassing seed germination, root morphogenesis, and floral induction (Toribio et al., 2020). All isolates secreted trans-zeatin, a type of cytokinin, enhancing plant growth, nutrient assimilation, and resilience under stress (Bayona-Morcillo et al., 2020; Uniyal et al., 2022). Notably, strains *N. punctiforme*, G23, and G35 were significant producers of ICA and IAA, auxins that promote root development and plant resilience (Wally et al., 2013). *Nostocales* cyanobacteria have been described to produce large amounts of IAA from tryptophan, a fact that could be related to their symbiotic activity in plants (Sergeeva et al., 2002; Hussain et al., 2015). Furthermore, gibberellin GA3 was identified in every evaluated strain, underscoring its ubiquitous role in plant growth and development (Hedden and Thomas, 2012; Amin et al., 2009). This aspect of cyanobacteria, particularly their ability to produce phytohormones, analogous to the plant-derived modulators, can promote plant growth and presents a significant opportunity for their application in agriculture (Mutale-Joan et al., 2023; Santini et al., 2021). The results from our study suggest that harnessing the unique capabilities of these cyanobacteria could lead to more sustainable and efficient cultivation practices, especially for crops like cotton, which are essential to global agriculture yet often require intensive inputs.

This study revealed that all tested cyanobacterial strains exhibited siderophores production and nitrogenase activity, with strains G23 and G42 showing the highest levels of nitrogen fixation. This finding underscores the potential of these two strains as biofertilizers, capable of solubilize metals required for the nitrogenase enzyme and thus augmenting nitrogen fixation and soil nitrogen levels. No linear correlation was observed between the percentage of heterocysts and nitrogenase activity, since the strain with the highest percentage of heterocysts G35 is not the one with the highest nitrogenase activity. This suggests that other factors, beyond mere heterocyst count, play a crucial role in nitrogen fixation efficiency. Furthermore, the variance in nitrogenase activity among the different strains could indicate a diversity in nitrogen-fixing capabilities, which might be exploited to tailor biofertilization strategies to specific crops or soil conditions. Our study also reveals a significant correlation between the nitrogenase activity of cyanobacterial strains G23 and G42 and growth enhancement in cotton plants. Notably, strains exhibiting higher levels of nitrogenase activity,

particularly G42, were most effective in promoting cotton growth, suggesting a direct link between their nitrogen-fixing capacity and plant growth enhancement. G42, in particular, showed a pronounced positive impact on root development. The deployment of cyanobacterial strains with active nitrogenase could represent a sustainable and eco-friendly alternative to synthetic fertilizers, aligning with the broader goals of minimizing environmental impacts while maintaining or even enhancing agricultural productivity.

Furthermore, under conditions of iron limitation, all tested cyanobacterial strains demonstrated the capability to synthesize siderophores. These iron-binding molecules, known for their low molecular weight and high affinity for iron, not only benefit the microorganisms by facilitating iron uptake but also enhance iron availability in the rhizosphere for plant uptake, which is of paramount ecological significance due to iron's role in limiting primary productivity in many settings (Roque et al., 2023). In the soils used for our analysis, where iron is not the limiting nutrient, beneficial impact of cyanobacterial inoculation on plant growth is not directly linked to siderophore production, but might be critical in iron-limited soils. Future research should explore the effects of plant inoculation under explicitly iron-limited conditions to further elucidate this relationship.

Given the encouraging outcomes of our study, we advocate for expanded research into the biofertilization potential of cyanobacteria across diverse agricultural settings. Future studies should also delve into understanding the mechanisms underlying the beneficial interactions between plants and cyanobacteria. This knowledge is crucial for optimizing and successfully implementing cyanobacterial-based biofertilization strategies, thus promoting an environmentally friendly, economically viable, and effective approach to enhancing crop productivity.

5. Conclusions

This study demonstrated the dynamic nature of soil nutrient transformation and the rich microbial diversity in cotton cultivation soils, with a special focus on the role of cyanobacteria as potential biofertilizers. The bacteriome analysis showcased a complex microbial ecosystem, essential for soil health and plant growth. Crucially, the isolation and characterization of nitrogen-fixing cyanobacteria, particularly strains G23 and G42, revealed their significant potential in promoting cotton growth. These cyanobacterial strains not only showed high nitrogenase activity but also enhanced plant growth, pointing towards a sustainable alternative to conventional fertilization methods. The use of these strains as an alternative to chemical-synthetic nitrogen fertilizers not only supports cotton growth but also contributes to maintaining microbial balance in the environment. Implementing these cyanobacteria-based fertilizers aligns with sustainable agriculture principles and offers a means to mitigate the environmental impacts of synthetic agrochemicals.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.171533.

CRediT authorship contribution statement

Lucía Jiménez-Ríos: Writing – review & editing, Methodology, Investigation. **Alejandro Torrado:** Writing – review & editing, Investigation, Formal analysis, Data curation. **José Luis González-Pimentel:** Writing – review & editing, Investigation, Data curation. **Macarena Iniesta-Pallarés:** Writing – review & editing, Investigation. **Fernando P. Molina-Heredia:** Writing – review & editing. **Vicente Mariscal:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis, Conceptualization. **Consolación Álvarez:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization.

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.

Data availability

The dataset obtained by the high-throughput sequencing is publicly available at the repository National Center for Biotechnology Information Sequence Read Archive (NCBI SRA) under the accession number PRJNA1054016. Other data generated in this work will be made available on request.

Acknowledgements

Soil material was obtained according to the guidelines of the Nagoya protocol for the access to Spanish genetic resources for research use under royal decree 124/2017 of 24 February. Reference: ESNC26.

Funding

This work was supported by the Corporación Tecnológica de Andalucía under grant number BFE14300. M.I.P. was supported by a predoctoral contract from the University of Seville, under the VI PPIT-US program. A.T. was funded by a NextGenerationEU 'María Zambrano' grant from the University of Seville, grant number MZAMBRANO-2021-20002. C.A. is funded by Consejería de Transformación Económica, Industria, Conocimiento y Universidades from Junta de Andalucía, grant number ProyExcel_00298.

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