

Biostimulatory Effects of *Limnospira indica* on the Modulation of Rhizosphere and Endosphere Microbiota and its Impact on Plant Growth

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Abstract

Cyanobacteria-derived biostimulants can improve plant performance through microbiota modulation. We tested culture fractions of *L. indica* on tomato (*Solanum lycopersicum*), focusing on growth and root-associated communities. Nitrogen deprivation in *L. indica* cultures increased significantly polysaccharides content including EPS (EPS 0.05 % in N⁺ to 0.15 % in N⁻; protein/polysaccharide ratio : 8 in N⁺ to 0.5 in N⁻). Residual culture medium (CM) and biomass extract (BME) from nitrate-rich and nitrate-deficient cultures, were applied as soil amendment or foliar sprays. EPS-rich CM N⁻ significantly enhanced flower production (49.8%, *p-value* = 0.005), while foliar BME N⁻ increased fruit yield (*p-value* = 0.008). 16S rRNA profiling revealed distinct rhizosphere and endosphere shifts, including enrichment of plant growth-promoting genera (Acidovorax, Devosia, Massalia). Notably, foliar CM N⁻ reshaped root microbiota, suggesting EPS-mediated systemic effects on plant metabolism and root microbiome assembly. This highlights the residual culture media as a biostimulant, offering new economic advantages.

1 Introduction

The sustainable intensification of agriculture to meet global food demands while minimising environmental impact is one of the key challenges of the 21st century [1]. Conventional agricultural practices, which rely heavily on chemical fertilizers and pesticides, often lead to soil degradation, biodiversity loss, and environmental contamination [2]. These limitations highlight the urgent need for innovative approaches that can enhance crop productivity while preserving ecosystem integrity.

At the same time, space exploration poses additional requirements for autonomous and highly reliable food production, stimulating research in closed-loop life-support systems (Lasseur et al. 2010). Within this context, ESA's MELiSSA program has developed bioregenerative systems that recycle waste into valuable resources for crop production, including the cultivation of the cyanobacterium *Limnospira indica* (formerly Spirulina), a nutrient-dense food source rich in proteins, vitamins, antioxidants, and iron [3, 4, 5]. To further improve productivity and resilience without external inputs, MELiSSA also explores microbial-based biostimulants, substances or microorganisms that stimulate

plant processes to enhance nutrient use efficiency, abiotic stress tolerance, and crop quality [3, 6, 7].

Microbial biostimulants are attracting increasing attention for their ability to modulate plant-associated microbiota and deliver bioactive compounds, thereby improving growth, nutrient uptake, and stress resilience [3, 6, 7]. Their activity often relies on reshaping rhizosphere and root-associated communities, which play essential roles in plant health through processes such as nitrogen fixation, phosphate solubilisation, and the synthesis of phytohormones including auxins and gibberellins [8, 9]. Recent work has shown that biostimulant-induced modulation of these microbial networks can substantially enhance crop performance under both optimal and suboptimal conditions [10, 11, 12].

Delivery route is a critical determinant of biostimulant efficacy [13]. Soil amendment introduces compounds into the root zone, where they interact with the rhizosphere microbiota, influence nutrient cycling, and modulate root colonisation [14]. In contrast, foliar application delivers compounds directly to the leaf surface, allowing rapid uptake and signalling, with potential downstream effects on root physiology and exudation [15]. However, systematic comparisons between these routes remain scarce, especially under controlled dose-equivalent conditions.

Here, we investigate the effects of *L. indica*-derived fractions on *Solanum lycopersicum* (tomato), a major crop valued for its nutritional quality and widely used as a model in biostimulant research [16, 17, 18]. Tomato was chosen because polysaccharides such as chitosan have already been shown to exert biostimulant effects [19, 20, 21, 22], and because its well-characterized, highly dynamic microbiome is particularly sensitive to soil, developmental stage, and external inputs, making it an ideal system to assess biostimulant-driven shifts in plant performance and root-associated communities [23, 24, 25, 26].

Although cyanobacteria-based biostimulants are increasingly studied due to their bioactive metabolites such as polysaccharides [27], there remains a lack of mechanistic evidence linking *Limnospira*-derived fractions under different culture conditions to plant phenotypes, microbiome dynamics (rhizosphere *vs.* root), solution chemistry under different application routes [28, 29, 30, 31]. Importantly, culture conditions have been shown to markedly affect the biochemical composition of *L. indica*, particularly with respect to polysaccharide biosynthesis. By example, nitrate limitation induces a pronounced metabolic shift, leading to enhanced accumulation of polysaccharides, including extracellular exopolysaccharides (EPS) [3].

In this study, we address these gaps by (i) comparing two *L. indica*-derived fractions, culture media (CM) and biomass extracts (BME), produced under nitrate-sufficient (N^+) and nitrate-deficient (N^-) conditions; (ii) applying dose-equivalent treatments via soil amendment and foliar application; (iii) monitoring tomato growth traits (height, branching, leaf, flower, and fruit number) over time; and (iv) characterizing treatment- and time-dependent shifts in rhizosphere and root-associated microbiota using 16S rRNA gene sequencing, and their associations with plant performance.

2 Results

We conducted a comprehensive characterisation of the different extract solutions used for biostimulation experiments in order to understand their composition and properties, which can influence their effectiveness in promoting plant growth through direct intake of bioactive component from *L. indica* extracts or through microbiome impact. By analysing factors such as growth curves of *L. indica*, carbohydrate and protein content, as well as polysaccharide composition, we can assess the qualities of the biostimulant extracts, their impact on plants characteristics and the associated microbiome impact.

2.1 *L. indica* culture fraction characterisation

Limnospira indica growth curve in presence or absence of nitrate and associated carbohydrates and proteins content was evaluated.

L. indica presented the ability to grow in presence and absence of nitrate in its environment (Figure 1). However, we observe a higher optical density, revealing a more effective growth, when the environment is in presence of nitrate, and, therefore, optimal. Indeed, while the end of the exponential phase is reached at 20 days with an $OD_{750} = 3$ in N^+ condition, it is reached at 9 days with an $OD_{750} = 2$ only in N^- condition. Remaining NO_3^- in N^- condition is observed between days 0 and 5, corresponding to the remaining nitrate from the mother cultures.

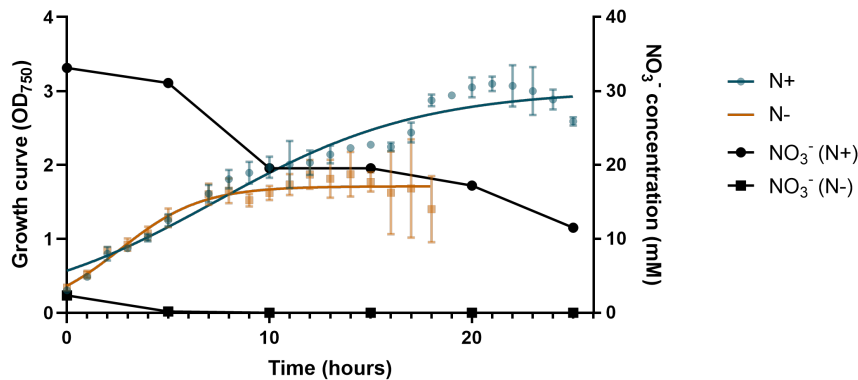


Figure 1: Growth curve of *Limnospira indica* PCC8005 in presence and absence of nitrate (N^+ in blue, N^- in orange) over 25 days. Nitrate concentration in both culture media is represented in black ($N^+ =$ black circle, $N^- =$ black square).

In addition, a total carbohydrates and proteins content was assessed to evaluate the impact and overall composition of *L. indica* when grown in presence (N^+) and absence (N^-) of nitrate at the end of the exponential growth with a respective OD_{750} (N^+) = 1.8 and OD_{750} (N^-) = 1.2 (Figure 2).

The protein content of *L. indica* when grown in presence of nitrate (N^+) was 76% of DW (Figure 2, A) while capsular and internal carbohydrates observed was 8% of DW (Figure 2, B). In parallel, when grown in absence of nitrate N^- , protein content of *L. indica* dropped to 31% of DW (Figure 2, A), and the capsular and internal carbohydrates content increased to 57% of DW (Figure 2, B). Last,

the amount of excreted polysaccharides is increased by a factor of 3 when grown in absence of nitrate from 0.05 % of DW in N⁺ to 0.15 % DW in N⁻ (Figure 2, C).

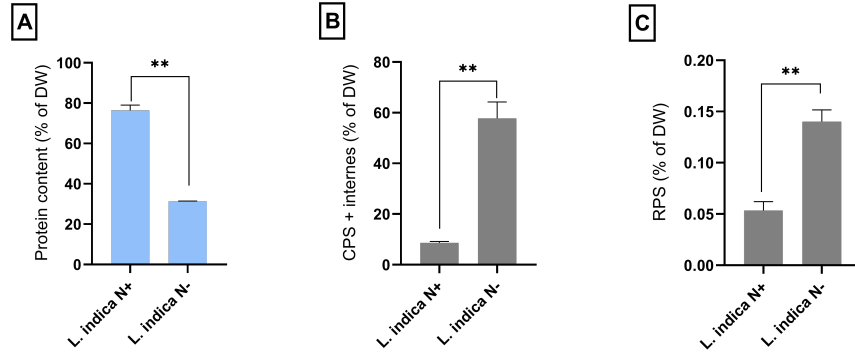


Figure 2: Protein and Polysaccharides content. (A) Protein content in percentage of biomass dried weight (% of DW). (B) Total carbohydrates content within the biomass (capsular and internal polysaccharides) in percentage of dried weight of biomass (% DW) (C) Total released carbohydrate content present in culture media (Released Polysaccharides), relative to dried weight of biomass. The values are shown as mean $\pm$ SEM. Statistical significance was tested using a t-test. $n=3$. DW = Dry Weight, CPS = Capsular polysaccharides, RPS = Released Polysaccharides. * = p - value < 0.05, ** = p - value < 0.01, *** = p - value < 0.001.

Numerous studies have investigated the role of nitrogen in the growth of *L. indica* [32, 33, 34, 35, 36]. One notable aspect of *L. indica*'s biology is its requirement for nitrogen in its environment, which has been shown to have a fundamental impact on its growth [37]. Indeed, it has been demonstrated that the absence of nitrate in the culture media can significantly impair the growth of *L. indica* [32, 36]. This is likely due to the crucial role that nitrogen plays in the metabolic processes and cellular development of cyanobacteria [38]. Removing the combined nitrogen sources in non-diazotrophic cyanobacteria has an impact on the culture growth [38]. Moreover, in nitrogen-deprived conditions, photosynthesis persists, leading to the degradation of phycocyanin as a nitrogen source [39]. The reduction in chlorophyll, phycocyanin and phycobilisome (PBS) content causes a noticeable shift in cell pigmentation, transitioning from the typical blue-green to a yellow-green hue, a process referred to as bleaching or chlorosis [39, 40]. Concurrently, carbon is accumulated in the form of both extra-cellular and intracellular polysaccharides, with glycogen serving as the primary intracellular storage compound [41, 42, 43, 44]. This accumulation reaches a level where cellular sedimentation occurs due to increased biomass density. Additionally, it has been observed that nitrogen stress can lead to the modulation of secondary metabolite production in *L. indica*, which in turn can affect its overall growth and development [45, 46, 47].

The importance of nitrogen for the growth of *L. indica* is further underscored by the fact that it is an essential nutrient for a wide range of species, including plants used in agricultural and horticultural applications [48, 3]. Nitrogen is a crucial component of various biomolecules, such as proteins, nucleic acids, and chlorophyll, all of which play vital roles in the growth and development of photosynthetic organisms [49].

The composition of internal and excreted polysaccharides was further evaluated to better characterise their monosaccharide profiles under nitrate-sufficient and nitrate-deficient conditions.

To evaluate the composition of polysaccharides synthesised by *L. indica*, the monosaccharide profiles of both intracellular (biomass-associated, capsular and internal) and extracellular (released into the culture medium) fractions were analysed. The relative abundances of monosaccharides were determined after acid hydrolysis and analysis through mass spectrometry (Figure 3).

Overall, the intracellular polysaccharides were dominated by Glucose and Galactose, which constituted the majority of the carbohydrate fraction within the biomass of N^+ and N^- conditions with respectively 58% and 97% of detected monosaccharides. Other monosaccharides, including arabinose+xylose, fructose+rhamnose, ribose and mannose, were detected and mainly present in BME N^+ condition. Within the BME N^+ arabinose+xylose were present at 7%, fucose+rhamnose at 23%, ribose at 10% and mannose at 6%. In parallel, BME N^- was composed of arabinose+xylose and fucose+rhamnose at respectively 1%, 2% each. Ribose and Mannose were not detected.

The extracellular polysaccharides exhibited a distinct composition. Glucose and galactose remains the most observed monosaccharides in both N^+ and N^- conditions with respectively 55% and 29% of detected monosaccharides. Fucose+rhamnose were more represented compared to their proportion in the biomass with 42% and 56% in N^+ and N^- respectively. Arabinose+xylose and ribose were less abundant in CM N^+ present at 3% and 0% respectively compared to the biomass (N^+) while they were more abundant in CM N^- present at 13% and 1% respectively compared to the biomass (N^-).

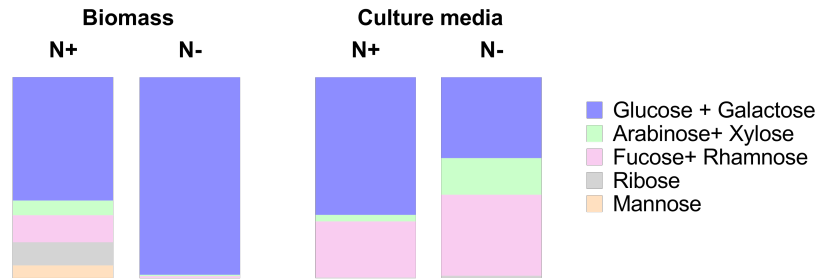


Figure 3: Relative composition in monosaccharides. Left, internal (intracellular and capsular) polysaccharides within *Limnospira indica*'s biomass (BME N^+ and BME N^-) and right, extracellular (excreted) polysaccharides within *Limnospira indica*'s culture media (CM N^+ and CM N^-). $n=5$, mean of replicates.

The monosaccharide composition observed in nitrate depleted biomass by [50] was also primarily composed of glucose and galactose in the control (N^+) (52%) or by [51] with 60%. However, [50] reported a higher concentration of rhamnose (30%) against only 9% by [51]. In contrast, under nitrate starvation, glucose remained the predominant monosaccharide in both studies (83% for [50] and 94% for [51]), aligning with our results. Similarly, [52] found that glucose was the only monosaccharide detected in nitrate-starved *Arthrospira* biomass.

As a result, polysaccharides from internal and excreted polysaccharides were mainly composed of glucose and galactose. Other detected monosaccharides showed a more balanced distribution. These findings indicate that *L. indica* modulates its extracellular polysaccharide composition, in response to environmental factors leading to a metabolic shifts due to the C:N ratio in nitrate deficient environment as observed in several cyanobacterial strains [53]. Nitrate is retrieved from photosynthesis-linked proteins (phycobilisom, phycocyanin, chlorophyll) and carbon is accumulated

in the form of both extracellular and intracellular polysaccharides [42, 40, 44]. Therefore, a decrease in nitrogen availability alters the C:N ratio, resulting in an excess of carbon. This excess carbon is subsequently utilised in the synthesis of polymers, including exopolysaccharides (EPS). Previous studies have shown that EPS production is particularly enhanced when the C:N ratio reaches 10:1 [53, 54].

2.2 Biostimulation of *Solanum lycopersicum*

Different parameters were evaluated in order to characterise plant growth and biostimulant solutions impact. These parameters included the total height of the plant (Figure 4), the number of ramifications (Supplementary Figure 1), leaves (Supplementary Figure 1), flowers (Figure 5, A and B) and early fruits (Figure 5, C and D). Importantly, the suckers on the plants were not removed during the observation period. Furthermore, the count of suckers was found to not be significantly different across the plants, indicating a uniform pattern of suckers development and direct impact on other parameters.

Regarding the plants size (Figure 4), when the first 20 days does not present a clear impact of the biostimulant extracts used on the plant, the observations made at day 35 shows significant differences between the conditions tested. Solutions prepared from *L. indica* biomass grown in absence of nitrates (BME N⁻) increased plant height relative to water control when used as watering solution (amendment) compared to the water control (Figure 4, C) while culture media from *L. indica* grown in absence of nitrates (CM N⁻) induced a diminution of *S. lycopersicum* size. In parallel, culture media of *L. indica* grown in presence of nitrates (CM N⁺) appeared to significantly increase the plant size when applied directly on the leaves compared to the water control while *L. indica* biomass grown in presence of nitrates (BME N⁺) induced an inhibition of the plant size.

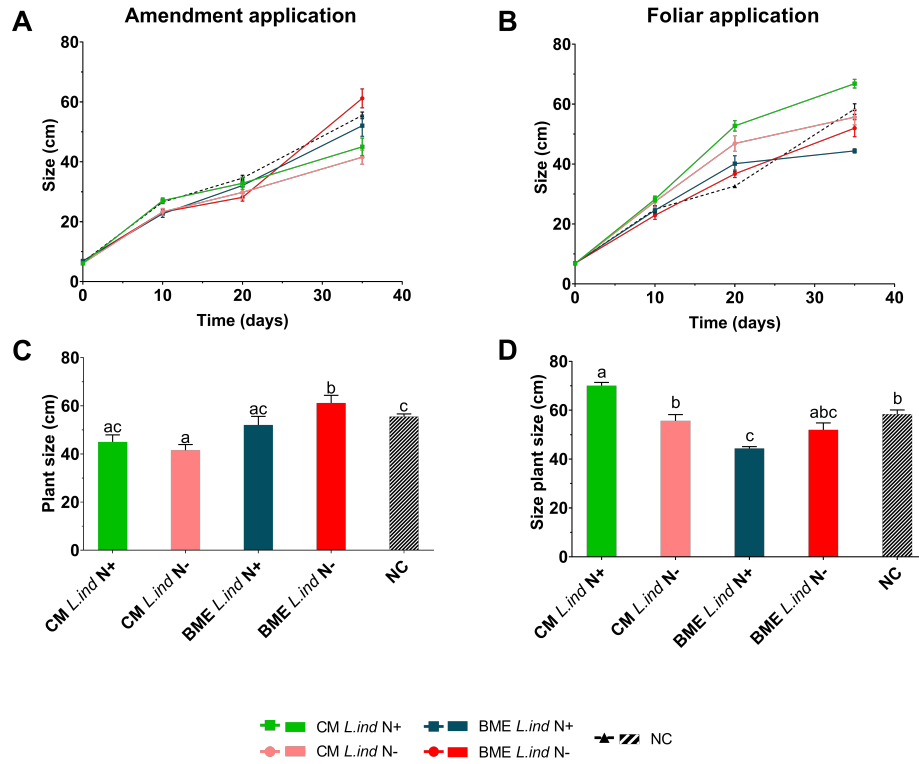


Figure 4: *Solanum lycopersicum* size (in cm) evolution under amendment (A and C) and foliar (B and D) biostimulant applications over 35 days (A and B) and at day 35 (C and D). The values are shown as mean $\pm$ SEM. Statistical significance was tested using a t-test, $n=5$. CM = culture media biostimulant, BME = biomass extract biostimulant, NC = negative control

When looking at the ramification and the leaves (Supplementary Figure 1, A) of *S. lycopersicum* (Supplementary Figure 1, C), it is observed that the amendment administration does not impact the number of ramification nor leaves with any of the biostimulant solutions tested. However, when applied through foliar application process (Supplementary Figure 1, B and D), biomass extract of *L. indica* grown in presence (BME N⁺) and absence of nitrates (BME N⁻) were shown to inhibit the number of ramifications observed and therefore the leaves compared to other biostimulant solutions.

In parallel, the number of flowers has been observed after 35 days of growth and revealed an interesting effect of biostimulant on *S. lycopersicum* (Figure 5). Indeed, while applied by amendment, culture media of *L. indica* grown in absence of nitrate (CM N⁻) has a significant impact on *S. lycopersicum* flowers production (Figure 5, A) compared to the other biostimulant solutions including water control. However, foliar application of biostimulant solutions did not present any difference in term of flower production amongst all conditions tested (Figure 5, B). Last, the number of fruits has been observed after 35 days of growth and revealed an interesting effect of biostimulant on *S. lycopersicum* (Figure 5). Indeed, when applied through amendment application, early fruits production were observed only with CM N⁻ biostimulation (Figure 5, C). In addition, when applied through foliar application, early fruits were observed in 3 biostimulant conditions: CM N⁻, BME N⁺, and BME N⁻, where only N⁻ solutions presented a significant increase of fruits early phenology (Figure 5, D).

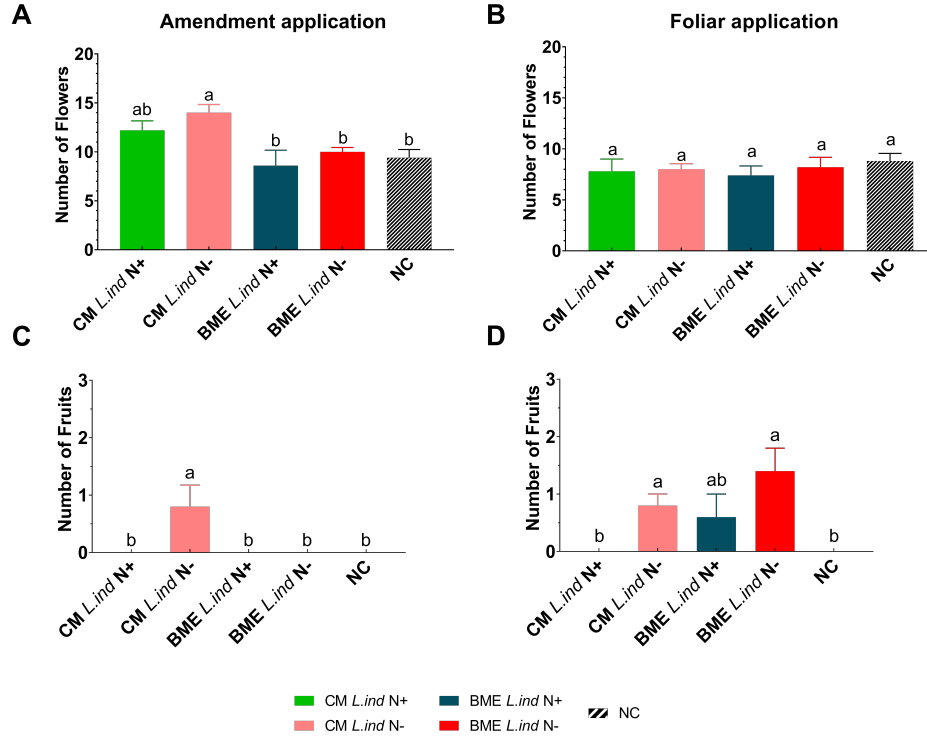


Figure 5: *Solanum lycopersicum* number of flowers (A and B) and fruits (C and D) under amendment (A and C) and foliar (B and D) biostimulant application after 35 days. The values are shown as mean $\pm$ SEM. Statistical significance was tested using a t-test, $n=5$. CM = culture media, BME = biomass extract, NC = negative control

Based on these results, it appears that the application of *L. indica* present a great interest for biostimulation. More precisely, CM N⁻ biostimulant presents interesting results both in amendment and foliar application. The flowering stage of the plant seems to be particularly impacted by the application of CM N⁻ and BME N⁻ biostimulants where the amendment application of CM N⁻ significantly increased the number of flower produced by *S. lycopersicum*. Following the flowering stage, the early reproductive phenology (fruits) seems to be increased by both applications with a maximal impact with BME N⁻ applied on the leaves.

2.3 Biostimulant impact on rhizosphere and endosphere microbiota over time

Samples analysed in targeted genomics were samples extracted from growth plants experiment at T0 (0 day), T20 (20 days) and T35 (35 days) of biostimulant application (Section 2.2).

The NMDS profile of the pairwise community dissimilarity (Supplementary Figure 2) demonstrated clustering patterns for each experiment based on the batch in which the experiment was conducted. Control groups were not consistently associated, indicating dissimilarities among them, and thereby not allowing comparison across the three experiments. Consequently, the results are structured across all eight datasets (Table 3) : CMRhizoA, CMRootsA, CMRhizoF, CMRootsF, BMRhizoA, BMRootsA, BMRhizoF, BMRootsF.

Although this dataset structure is not without limitations, it aligns with the study's focus on evaluating the differential impact of treatments between water control and culture derived fraction

of *L. indica* from N⁺ and N⁻ cultures (from both culture media and biomass extract). This structure facilitates testing the hypothesis: *Does biostimulant produced from nitrate deficient (N⁻) Limnospira indica culture significantly impact the microbiota, and therefore explain the phenotypic results observed in Section 2.2?*

Based on the phenotypic results obtained in *S. lycopersicum* seedlings growth experiments (Section 2.2), the following analysis is mainly focused on datasets of culture media (CM) analysis in presence (N⁺) and absence (N⁻) of nitrate and further referred as CMRhizoA, CMRootsA, CMRhizoF, CMRootsF. Indeed, the primary interest in biostimulants lies in their impact on flowers and fruits production rather than on plant size. Datasets of biomass extract (BME) analysis are presented in supplementary data for clarity yet being discussed in the following sections.

The PERMANOVA analysis significantly highlighted the influence of both time and conditions for CM and BME treated samples (conditions * time) on the bacterial community structure in all experiments conducted, within the rhizosphere and endosphere microbiota (Supplementary Table 1). When accounting for inter-batch variation using the strata argument, significant differences were also observed between biostimulant conditions (Supplementary Table 2). In amendment applications, CM N⁻ differed significantly from the negative control in both rhizosphere and endosphere microbiota (Supplementary Table 3). In contrast, for foliar applications, no differences were detected between conditions in both the rhizosphere and roots microbiota. The dissimilarity observed among control samples indicates that a degree of inter-batch variation is inherent to our system. While such variability may reduce the robustness of statistical inference and complicate the generalisation of treatment effects, the use of batch-constrained PERMANOVA helps mitigate this influence by restricting permutations within each batch. Importantly, the consistent detection of treatment-related differences across independent batches suggests that the observed effects are not artefacts of intra-batch variability but reflect reproducible biological responses. The α -diversity of the bacteria community of treated samples with biostimulant were measured via the Shannon index in the different conditions (Supplementary Figure 3). All statistics analysis (*p-values*) are presented in Supplementary data (Supplementary Table 4). The analysis revealed that a CM N⁻ amendment application induced a significant diminution of the α -diversity of the bacterial community in the rhizosphere microbiota at T20 compared to the water control (either at the same time and at T0). No difference was observed amongst all 3 conditions at T35 but a similar significant increase between T20 and T35 is noted in water control, CM N⁺ and CM N⁻ conditions. In parallel, the α -diversity of the bacteria community in endosphere microbiota, revealed a clear significant increase over time for all the conditions tested. When applied through foliar application the rhizosphere microbiota presented a significant increase from T0 to T35 in both CM N⁺ and CM N⁻ conditions. In the endospheric microbiota, we observed a significant increase over time (T0 - T20 - T35) in all conditions tested. At T35, the α -diversity is higher in CM N⁻ compared to water control and CM N⁺. Similar results are observed in samples biostimulated with biomass extract (N⁺ and N⁻) through both applications. In addition, the endosphere microbiota in foliar application of BME N⁺ and BME N⁻ presented an α -diversity higher at T35 BME N⁺ and BME N⁻.

Previous studies have shown similar evolution of the α -diversity over time. Indeed, potato plants

has shown a rhizosphere microbiota with a higher community diversity during the flowering stage, while the seedling and early stage showed a lower diversity [55, 56, 57]. Nevertheless, while the bacterial community has an impact on plant growth [58, 59], plant’s released metabolites also affect the microbial community both positively and negatively depending on the development stage of the plant and on the metabolites produced. Saccharides positively impact microbiome while antimicrobial compounds have an expected negative impact on the microbial diversity [60]. Regarding foliar application, it seems obvious that the microbial community evolution result from the metabolites’ production from the plant.

Alongside with α -diversity, the distinctness of the microbial communities highlight the impact of biostimulant on the said communities. Depending on the biostimulant used and application processes, the distinctness revealed the apparition of unique genera as shown on the Venn diagrams (Supplementary Figure 4) and resumed in Table 1. At T35, most of the observed genera are shared in all conditions tested (from 27% to 65%) apart from CMRhizoF where only 18% correspond to shared genera from all conditions. Nevertheless, regarding unique genera observed in each condition, more unique genera are observed in samples biostimulated with N⁻ solutions except CMRhizoA and BMRootsA in both treatments. Similar results are observed when biomass extract is used as biostimulant solution.

Table 1: Unique genera repartition by conditions in rhizosphere and endosphere microbiotes.

Biostimulant condition	Rhizosphere microbiota	Endosphere microbiota
CM - Amendment	Most unique genera observed in control condition, but observed genera are mainly common to all conditions.	Most unique genera observed in CM N ⁻ condition, but genera observed are mainly common to all conditions.
CM - Foliar	Most unique genera observed in CM N ⁻ condition.	Most unique genera observed in CM N ⁻ condition, but genera observed are mainly common to all conditions.
BME - Amendment	Most unique genera observed in BME N ⁻ condition, but genera observed are mainly common to all conditions.	Most unique genera observed in H ₂ O condition, but genera observed are mainly common to all conditions.
BME - Foliar	Most unique genera observed in BME N ⁻ condition, but genera observed are mainly common to all conditions.	Most unique genera observed in BME N ⁻ condition, but genera observed are mainly common to all conditions.

The application of microbial-based biostimulants has been shown to increase the abundance and diversity of bacteria involved in nutrient cycling, such as nitrogen-fixing bacteria and phosphate-solubilising bacteria [61]. This microbial distinctiveness likely contributes to the phenotypic variations observed in *S. lycopersicum* growth such as early reproductive phenology seen in Section 2.2.

Furthermore, the composition of microbiota phyla after 20 and 35 days of biostimulation, has been analysed and is detailed in supplementary data (Supplementary Figure 5 and Supplementary Figure 6). After a biostimulation with all conditions, the endospheric microbiota was dominated by four major taxa: α -, β -, γ -*proteobacteria*, and *Bacteroidota*. In the rhizosphere microbiota, the same four taxa were identified, in addition to *Firmicutes* and *Acidobacteria*. No bacterial group

was exclusive to any particular condition. Overall, *α-proteobacteria* and *β-proteobacteria* predominantly characterised the endosphere microbiota, while *α-proteobacteria* and *γ-proteobacteria* were more abundant in the rhizosphere. These patterns highlight shifts in dominant phyla and genera under biostimulation. Nevertheless, while these taxa are associated with plant growth-promoting functions in the literature, their roles in our system cannot be directly inferred from taxonomic enrichment alone, and such functional interpretations should be considered hypothesis-generating pending direct experimental validation. Indeed, this taxa combination has already been observed in *Arabidopsis thaliana* and potato plants [62, 55] and has already been described as impacting plant growth. *Alphaproteobacteria* are known to include plant growth-promoting bacteria (PGPB), with several genera recognised as symbionts in legumes [63, 64]. *Betaproteobacteria* play a key role in the nitrogen cycle, facilitating plant-available nitrogen forms while limiting CO₂ fixation. They promote plant growth by fixing nitrogen in various plant species and enable the oxidation of ammonium to nitrite and nitrate, an essential reaction for plant development [65]. *Gammaproteobacteria* also contribute to plant growth by producing compounds such as auxins, gibberellins, cytokinins, and ethylene, as well as having an impact on nitrogen fixation and mineral solubilisation [10, 63, 66]. Lastly, *Bacteroidota*, identified as plant growth-promoting rhizobacteria (PGPR), demonstrates an ability to colonise plant roots successfully. Although their specific effects on plants are yet to be better described, they are known to significantly colonise during early plant growth stage [67, 68]. Further metagenomic analysis or assays (*e.g.*, *nifH* qPCR, enzyme activities) would be required to directly validate the functional roles of these taxa in our system.

The 10 most represented genera of each CM biostimulated conditions are displayed in the Circos graphs (Figure 6) where relationships between different genera and treatment conditions are shown over time.

In both the CMRhizoA and CMRhizoF experiments, the four predominant genera are *Rhodanobacterium*, *Devosia*, *Chitinophaga*, and *Dokdonella*. These four key genera in the biostimulation profiles have well-known plant growth-promoting properties. *Rhodanobacter* was prominent across both amendment and foliar treatments with its relative abundance comparable between amendment and foliar treatments. This genus has been recognised for its plant growth-promoting properties, particularly as an endophytic bacterium in *Solanum lycopersicum* where it enhances biotic and abiotic stress tolerance [69]. Additionally, *Rhodanobacter* is a nitrogen-fixing bacterium [70] that produces indoleacetic acid (IAA), which stimulates root growth, thereby facilitating nitrogen uptake from the soil [71, 70]. This ability to promote nitrogen and carbon recycling within the soil ecosystem further underscores its ecological significance and potential applications in sustainable agriculture [72]. Our study revealed a similar relative abundance of $18 \pm 1\%$ of *Rhodanobacter* at T0 and T20 in all conditions except when CM N⁻ was applied through foliar application where the relative abundance dropped at 13%. Furthermore, *Rhodanobacter* relative abundance decreased at $13 \pm 2\%$ at T35 except when CM N⁻ in both applications, where it dropped at 10%. Its abundance mainly in the first days of our experiment revealed its beneficial impact on the early plant growth through plant nitrogen fixation and stress tolerance. Its decline by T35, particularly under CM N⁻ conditions, reflects its temporal appearance within the microbial community.

Similarly, *Devosia* has shown an equivalent abundance across biostimulant treatments, suggesting

its involvement in root development and nitrogen cycling throughout the experiment. Known for its nitrogen-fixing capabilities [70], *Devosia* contributes to plant growth by possibly producing phytohormones that enhance root development. This genus has been linked with increased plant growth, improved mycorrhization, and nitrogen accumulation [73], suggesting its promising role in enhancing crop productivity [74]. Our study presented an initial increase of *Devosia* relative abundance from T0 (2% in CMRhizoA and 1% in CMRhizoF) to T20 ($5\pm 1\%$), displaying stable abundance from T20 onwards across treatment conditions.

Chitinophaga exhibited a significant decline from T0 to T35. The sharp decline over time of some genera, such as *Chitinophaga* could reveal a competitive interaction within the rhizosphere, as previously linked to reduced bacterial α -diversity. Previous studies have demonstrated that the presence of *Ralstonia solanacearum* in the rhizosphere can reduce the α -diversity of the bacterial community, with genera like *Bacillus* and *Chitinophaga* being negatively impacted [58]. This decline in *Chitinophaga* abundance under certain conditions may thus be indicative of competitive or antagonistic interactions within the rhizosphere microbial community. Our studies highlighted a relative abundance of *Chitinophaga* of 6% in CMRhizoA and 15% in CMRhizoF decreasing drastically at $4\pm 1\%$ at T20, and 3% at T35 in all conditions of both experiments.

Lastly, *Dokdonella* displayed slightly different results between foliar and amendment application, with increased abundance over time in specific conditions, pointing to its potential sensitivity to biostimulants. *Dokdonella* species are aerobic rods isolated from soil, and certain species within this genus can perform aerobic nitrate reduction, which may play a role in soil nutrient cycling [75]. Our study has exhibited an increase of relative abundance from T0 (2%) to T20 (4%), followed by stable relative abundance through T35 in amendment application. When the biostimulants were applied through foliar application, the water control revealed an increase from T0 (3%) to T35 (8%). This increase was also observed in CM N⁺ with a maximum of 6%. CM N⁻ condition relative abundance appeared to increase as others at T20 (6%) but a slight decrease to T35 (4%) was observed.

Overall, the presence and behaviour of these bacterial genera in the biostimulation context reveal their roles in nutrient cycling, stress tolerance, and plant growth promotion. The temporal evolution of the relative abundance of bacterial genera in this study highlights dynamic shifts in microbial community composition under biostimulation conditions. This study highlights the dynamic behaviour of key plant growth-promoting bacterial genera (*Rhodanobacter*, *Devosia*, *Chitinophaga*, and *Dokdonella*) under biostimulation conditions with amendment and foliar treatments over time. The observed microbial dynamics provide insights into how biostimulants can shape the rhizosphere microbiome and optimise conditions for sustainable agricultural practices.

In both the CMRootsA and CMRootsF experiments, the five predominant genera of interest are *Massilia*, *Rhodanobacter*, *Acidovorax*, *Shinella*, and *Asticcacaulis*. Similar to observations in the rhizosphere, these genera are recognised for their plant growth-promoting properties and are frequently highlighted in biostimulation studies for their beneficial effects.

The relative abundance evolution over time of *Massilia* appears similar between amendment and foliar treatments. This genus is associated with plant growth promotion, particularly in stressful environments. *Massilia* has been shown to enhance plant resilience to salinity stress helping with nu-

trient accumulation and root colonisation in corn plants [76]. Additionally, *Massilia* has been linked to the promotion of lateral root growth and biomass accumulation, especially in flowering plants like maize, where it associates with root functional genes involved in reproduction [77]. Our study revealed a marked decrease of *Massilia* relative abundance over time starting with a high relative abundance of $32 \pm 2\%$ at T0 decreasing at $13 \pm 2\%$ at T20 until $3 \pm 1\%$ at T35 in both application methods. Therefore, considering previous studies on this genus, its presence could suggest a shift in the plant growth stage with the arrival of the flowering stage. In addition, the presence of *Massilia* in the microbial community may also be beneficial in supporting early plant growth, as suggested by co-occurrence with protists that positively impact the bacterial community structure and nitrogen uptake [78, 79, 80] suggesting its importance at early growth stage.

Rhodanobacter, a well-known plant growth promoter [69, 70] exhibited a higher relative abundance in amendment treatments compared to foliar treatments, with a similar temporal pattern in both. *Rhodanobacter* has shown a high initial relative abundance at T0 (15% in CMRootsA and 9% in CMRootsF) and decreased strongly at T20 (7% in CMRootsA and 0.4% in CMRootsF). At T35, *Rhodanobacter* relative abundance was in both treatments at $3 \pm 1\%$.

Acidovorax displayed a distinct response depending on the biostimulant application method. While this genus is traditionally known as a biotrophic pathogen, several *Acidovorax* strains are commensal or beneficial, producing hormones and secondary metabolites that promote plant growth and help to compete with pathogens [81, 82]. In the amendment experiment (CMRootsA), *Acidovorax* relative abundance remained stable from T0 to T20 ($5 \pm 1\%$), whereas it increased with the application of CM N⁺ and CM N⁻ biostimulants at T35, reaching respectively 9% and 7%. On the contrary, in the foliar treatment, *Acidovorax* abundance decreased over time, starting from 11% decreasing at $7 \pm 1\%$ in water control and CM N⁺. Especially low levels have been observed at T20 (0.3%) and T35 (1%) in the CM N⁻ condition relative to other treatments.

As observed with *Acidovorax*, the relative abundance of *Shinella* presented a distinct response depending on the biostimulant application method. In the amendment experiment with water control, *Shinella* abundance decreased steadily from T20 (6%) to T35 (1%), while it showed a slight increase at T20 (9%) followed by a decrease at T35 (6%) in the CM N⁺ and CM N⁻ conditions. A similar pattern was observed in the foliar treatment with all biostimulant conditions with a relative abundance of 1% at T0, increasing to $7 \pm 2\%$ at T20 and finally decreasing of $3 \pm 1\%$. Known for its production of phytohormones, *Shinella* can positively influence plant growth and development by enhancing root formation and seed germination [83, 84]. In particular, strains of *Shinella* have been shown to mitigate microcystin (MC) accumulation in plants, reducing toxin levels in roots and eliminating it from leaves and fruits, which suggests potential applications for enhancing plant safety in contaminated environments [85]. In our study, this behaviour could derive from competitiveness of microbial communities exacerbated by the biostimulation treatments.

Finally, *Asticcacaulis*'s relative abundance showed distinctive behaviour based on the biostimulant applied but presented similar results when applied on the soil or leaves. This genus has been identified as a plant growth-promoting bacterium in crops like sugar beet, where it enhances seedling growth and supports biofilm formation that can protect plants against environmental stress and diseases [86, 87]. Indeed, *Asticcacaulis* showed an increase in abundance in the control from T0 (2%) to T35 ($5.5 \pm 1.5\%$) but a decrease from T20 (6%) to T35 (3%) in the CM N⁺A, CM N⁺F and CM

N⁺A conditions. Interestingly, CM N⁺F biostimulants made the relative abundance of *Asticcacaulis* stable at 3% at T20 and T35. The higher abundance of *Asticcacaulis* in amendment-treated roots may suggest that this application method is more valuable to supporting beneficial biofilms in the rhizosphere.

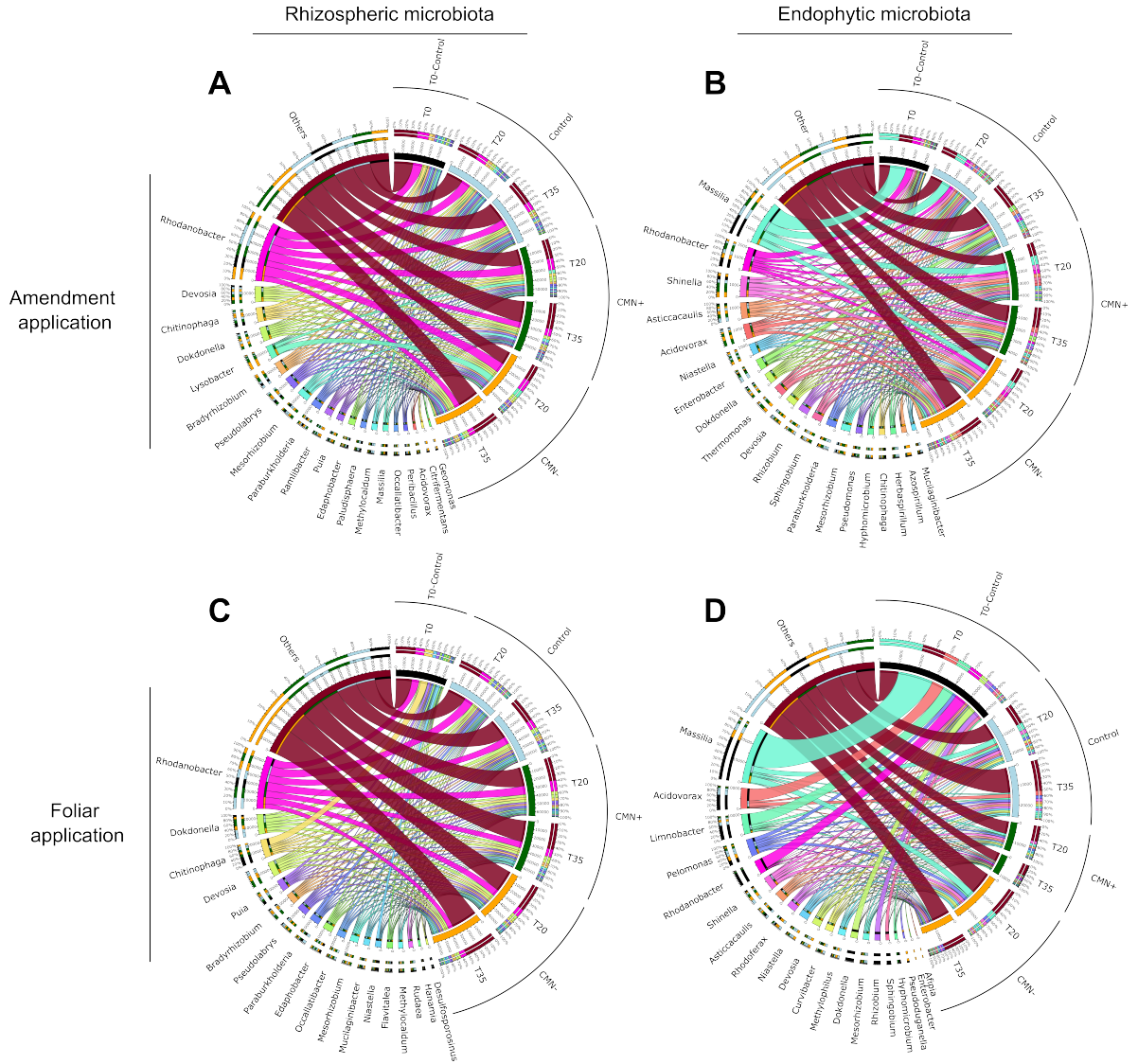


Figure 6: Rhizospheric and endospheric microbiota modulation depending on biostimulant and application. Circos graphs [88] representing the relative abundance of rhizosphere microbiota (A and C) and endosphere microbiota (B and D) at T0, T20 and T35 following the application of *L. indica* culture media-based biostimulant via amendment (A and B) and foliar (C and D) treatment. $n_{\text{per treatment}}=5$, $n_{\text{per plant}}=5$

The results provide a detailed characterisation of the genera identified within the rhizosphere and endosphere microbiota following biostimulation with *L. indica* culture media as a biostimulant. These findings indicate that biostimulation with culture media and its excreted metabolites, mainly polysaccharides, may favour specific genera in the plant microbiota contributing to enhanced plant growth. These genera has been also observed in the microbiota of plants biostimulated with biomass extract from *L. indica* (Supplementary Figure 7). A similar distribution and evolution of these genus has been similar in rhizosphere microbiota. However, roots microbiota appeared to differ from results observed with CM biostimulant. *Massalia* was the most impacted genus with an important decrease of the relative abundance from T0 to T35 (23% to 11% in amendment and 41% to 26% in foliar application) with water control, and an important increase to 45% in CM N⁺ when applied on the

leaves and CM N⁻ when applied on the soil at T35. These results underscore the significance of genera-specific abundance in the plant growth promotion process, genera that are overly abundant or, on the contrary, less prevalent may diminish the beneficial impact on plant growth promotion.

To further delve into biostimulation impact on genera in the microbiota of both rhizosphere and roots, heatmaps were realised for each experiment (Figure 7 and Figure 8: RhizoA, RootsA, RhizoF, RootsF). These heatmaps displayed respectively 95 genus in five RGs, 63 genus in four RGs, 43 genus in four RGs and 52 genus in five RGs statistically significantly affected identified using nbGLM analysis.

In the CMRhizoA experiment (Figure 7, A), RG1 comprise genera predominantly found at T0 with an increased prevalence at T20 under CM N⁺ and CM N⁻ conditions. RG2 included genera primarily present at T20 in CM N⁺ and CM N⁻. RG3 included genera found consistently in controls (all time points), as well as in CM N⁺ and CM N⁻ at T35. Within RG4, the most representative genera were observed across all conditions at T35. Finally, RG5 was characterised by genera present under all conditions at T35 but more predominantly in CM N⁻ condition.

In the CMRhizoF experiment (Figure 8, A), RG1 comprise genera predominantly found in all conditions at T20 and T35. RG2 comprises genera present at T35 in all conditions and slightly over represented in CM N⁻. RG3 included genera found at T20 in all conditions. Finally, in RG4, the most representative genera were observed at T0.

In the CMRootsA experiment (Figure 7, B), RG1 comprises genera predominantly found in control at T35. RG2 is composed by genera present at T35 in all conditions and over presented in CM N⁻. RG3 included genera found massively at T0. Finally, in RG4, the most representative genera were observed across all time points in CM N⁺ and CM N⁻.

In the CMRootsF experiment (Figure 8, B), RG1 comprises genera predominantly found in all conditions at T20 and T35. RG2 is composed of genera present at T35 in water control and CM N⁻ conditions. RG3 included genera found only at T0. RG4 included a single genus, *Bosea*, found in CM N⁺ condition at T35. Finally, in RG5, the most representative genera were massively observed at T35 in CM N⁻ condition.

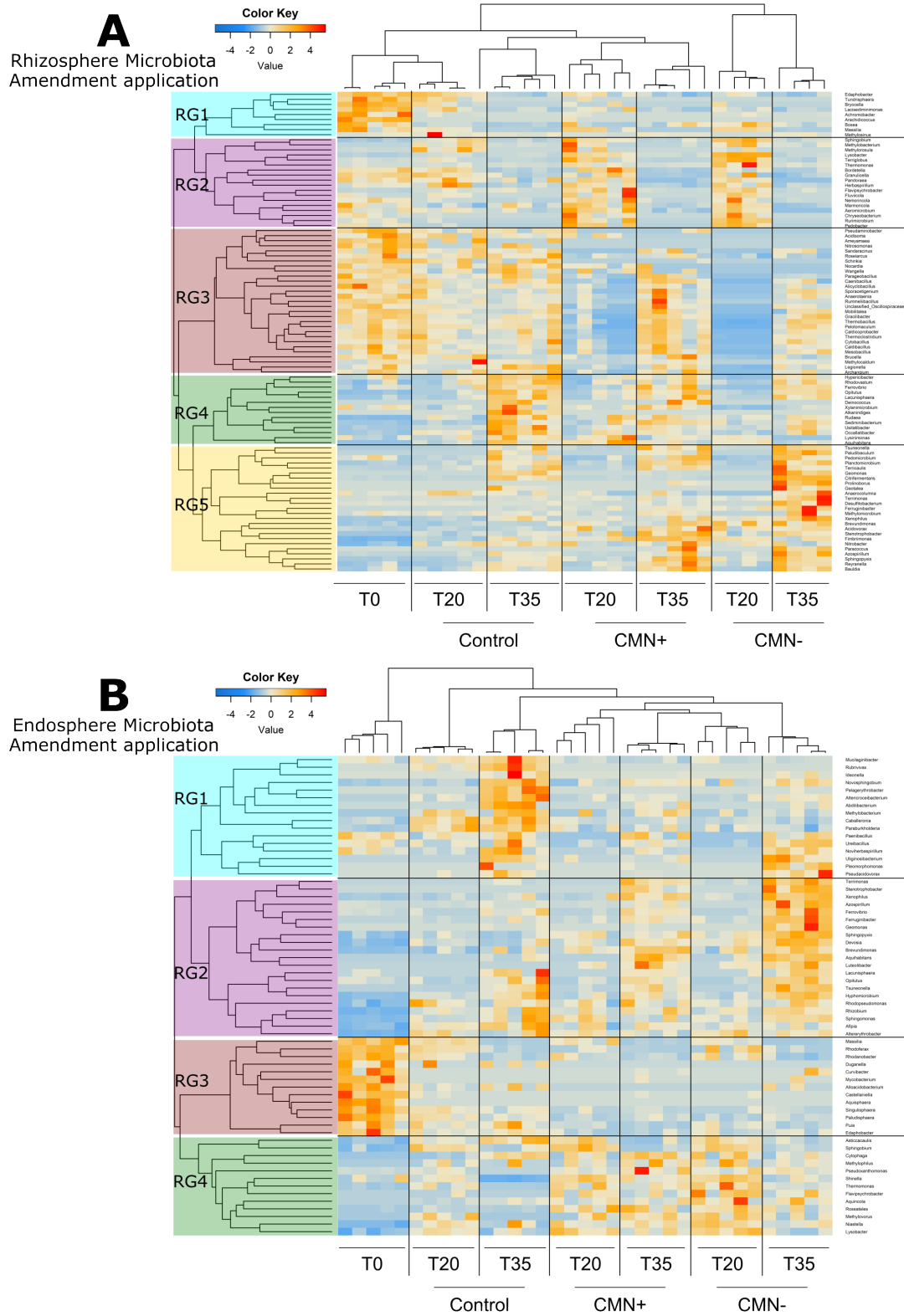


Figure 7: Heatmaps of the genera from the rhizosphere microbiota (A) and endosphere microbiota (B) after amendment treatment. Genera represented are significantly affected at T0, T20 and T35 following the application of *L. indica* culture media based biostimulant via amendment treatment. RG = Response Group. $n_{\text{per treatment}}=5$, $n_{\text{per plant}}=5$

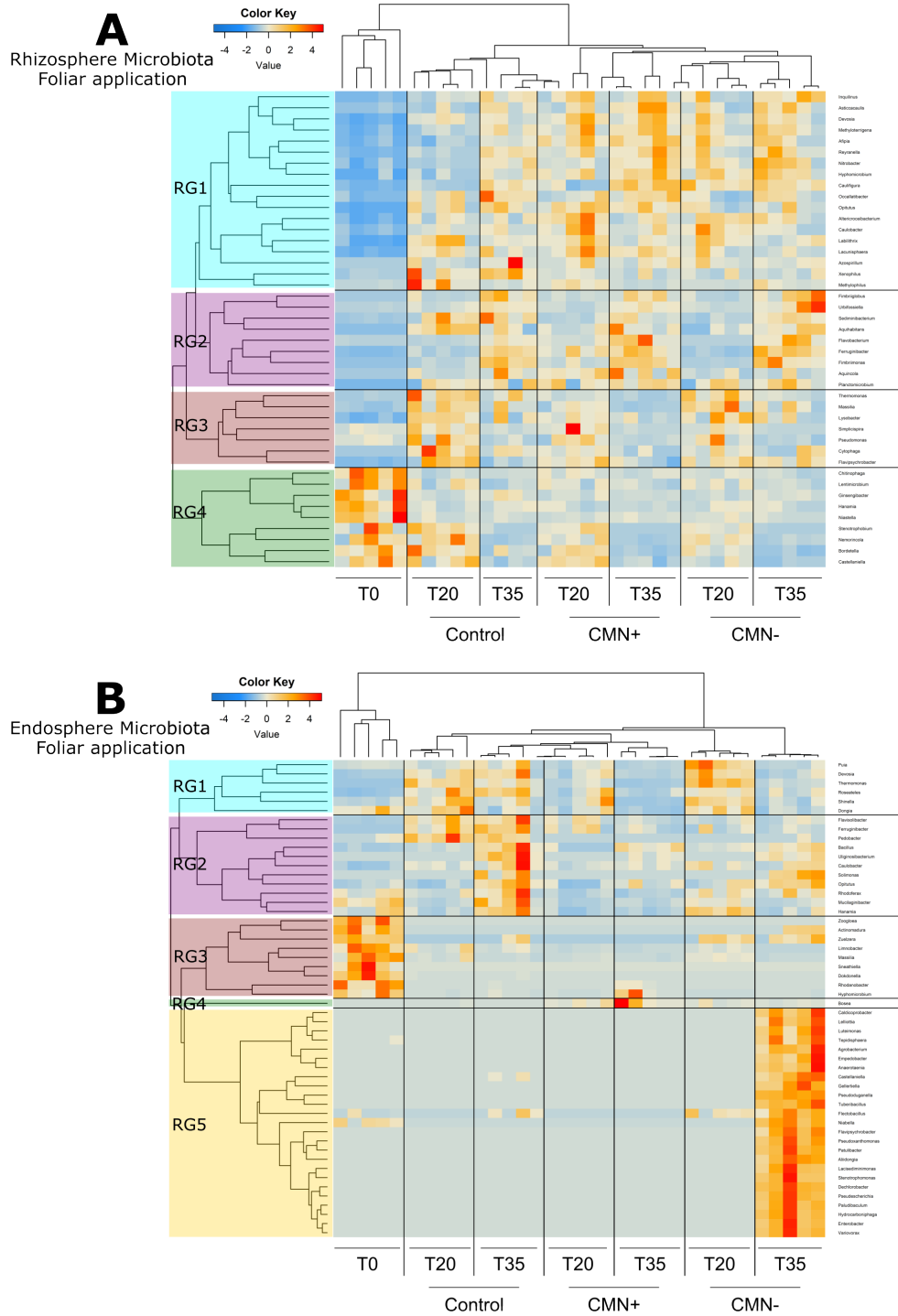


Figure 8: Heatmaps of the genera from the rhizosphere microbiote (A) and endosphere microbiote (B) after foliar biostimulant application. Genera represented are significantly affected at T0, T20 and T35 following the application of *L. indica* culture media based biostimulant via foliar treatment. RG = Response Group. $n_{\text{per treatment}}=5$, $n_{\text{per plant}}=5$

Our analysis revealed that when CM from *L. indica* used as biostimulants, several genera are prevalent compared to the water control groups over time. Overly expressed genera appeared in biostimulated conditions with CM N⁻ biostimulant at T35. Amongst them genus were particularly highlighted *Acidovorax*, *Aeromicrobium*, *Agrobacterium*, *Aliidongia*, *Anaerocolumna*, *Anaerotaenia*, *Azospirillum*, *Bauldia*, *Brevundimonas*, *Caldicoprobacter*, *Castellaniella*, *Chryseobacterium*, *Citrifermentans*, *Dechlorobacter*, *Desulfitobacterium*, *Devosia*, *Empedobacter*, *Enterobacter*, *Ferrovibrio*, *Fer-*

ruginibacter, *Ferruginibacter*, *Fimbriglobus*, *Fimbriimonas*, *Flavipsychrobacter*, *Flavobacterium*, *Flectobacillus*, *Fluviicola*, *Gellertiella*, *Geomonas*, *Geotalea*, *Hydrocarboniphaga*, *Lacisediminimonas*, *Lelliottia*, *Luteimonas*, *Lysobacter*, *Methylobacterium*, *Methyломicrobium*, *Nemorincola*, *Niabella*, *Nitrobacter*, *Paludibaculum*, *Paracoccus*, *Patulibacter*, *Pedobacter*, *Pedomicrobium*, *Prolinoborus*, *Pseudoscherichia*, *Pseudoduganella*, *Pseudoxanthomonas*, *Reyranella*, *Rurimicrobium*, *Sphingopyxis*, *Stenotrophobacter*, *Stenotrophomonas*, *Tepidisphaera*, *Terrimonas*, *Tsuneonella*, *Tuberibacillus*, *Urbifossiella*, *Variovorax*, and *Xenophilus*. Most of these genera has been observed in tomato rhizosphere or endosphere microbiota in previous studies [10, 89]. Additionally, a large proportion of these genera were also characterised as impacting during tomato flowering, which corresponds to our T35 [10]. Genus revealed by the heatmaps at T35 in CM N⁻ conditions (both applications) were statistically different in endosphere microbiota compared to the rhizosphere microbiota (Figure 7, B and Figure 8, B). More importantly, RGs highly represented in this analysis corresponded to genera present in N⁻ conditions. These genera are represented in the Figure 9.

A clear increase of *Pseudoduganella* and *Afipia* is observed in case of a foliar application of CM N⁻ (Figure 9). These gram negative bacteria are known for their potential as plant growth promoters [90, 71]. In the meantime, *Enterobacter*, a known plant growth promoter [91, 92] presented a clear over abundance in CM N⁻ condition in foliar application compared to CM N⁺ biostimulant and water control with the same application where it is not represented at all. Nevertheless, *Enterobacter* was observed in amendment application (all conditions) but with a minimal abundance compared to CM N⁻ condition applied on the leaves. Last, *Variovorax* has been observed in majority in CM N⁺ condition with foliar application. Its abundance was reduced in CM N⁻ condition with foliar application and even less present in all conditions represented in Figure 9.

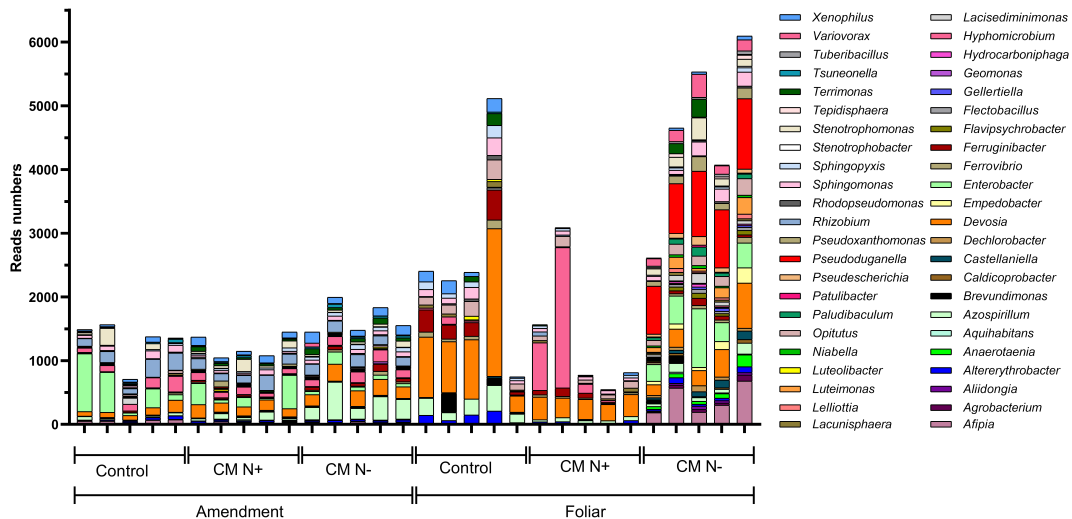


Figure 9: Abundance of 43 genera highlighted by the Heatmaps at T35 in CM N⁻ conditions in both amendment and foliar treatments. CM = Culture Media biostimulant, N⁻ = nitrate deficient culture, N⁺ = nitrate sufficient culture.

Amongst the genera observed in Figure 9, the most significantly abundant genera observed in CM N⁻ with amendment or foliar treatment are represented in Table 2 with their known impact on plant growth.

Table 2: Summary of genera significantly impacted by biostimulant from *Limnospira indica* CM N⁻ through amendment or foliar application at T35 and their impacts on plant growth.

Impact Category	Genus	Observed conditions	Mechanisms	References
Bioremediation	<i>Alidongia</i>	CMRootsF	Produces poly- β -hydroxybutyrate, contributing to biodegradable plastics.	[93]
Bioremediation	<i>Dechlorobacter</i>	CMRootsF	Reduces perchlorate contamination in the environment.	[94]
Bioremediation	<i>Desulfitobacterium</i>	CMRhizoA	Part of microbial communities that degrade chlorinated organics and form soluble metal sulfides, aiding in contaminated soil remediation.	[95, 96, 97]
Bioremediation	<i>Feruginibacter</i>	All	Found in wastewater treatment, secretes extracellular polymers, forming sludge flocs and biofilms.	[98]
Bioremediation	<i>Fimbrigiobus</i>	CMRhizoF	Exhibits chitinolytic and cellulolytic activities, useful in bioremediation.	[99, 100, 93]
Bioremediation	<i>Flectobacillus</i>	CMRootsF	Identified as possessing bioremediation properties.	[101]
Bioremediation	<i>Methylomicrobium</i>	CMRhizoA	Aerobic methanotrophs contributing to bioremediation.	[102]
Bioremediation	<i>Paludibaculum</i>	CMRhizoA, CMRootsF	Capable of dissimilatory ferric reduction, aiding in bioremediation.	[93, 103, 104]
Bioremediation	<i>Patulibacter</i>	CMRootsF	Degrades pharmaceutically active compounds like ibuprofen in soil environments.	[105]
Bioremediation	<i>Xenophilus</i>	CMRhizoA, CMRootsA	Produces siderophores and IAA, degrades azo dyes, and exhibits bioremediation properties.	[106]
Plant Growth Promoter	<i>Azospirillum</i>	CMRhizoA, CMRootsA, CMRhizoF	Demonstrates increased phytohormones production under favorable and stressful conditions, improving plant physiology and drought/salinity tolerance.	[107, 10, 93]
Plant Growth Promoter	<i>Caldicoprobacter</i>	CMRhizoA, CMRootsF	Proven to have a positive impact on plant growth and considered as PGPB through nitrogen fixation.	[10]
Plant Growth Promoter	<i>Devosia</i>	CMRhizoA, CMRootsA, CMRhizoF, CMRootsF	Promotes nitrogen fixation, produces phytohormones aiding root development, and enhances rice crop productivity through mycorrhization and nitrogen accumulation.	[70, 73, 74]
Plant Growth Promoter	<i>Empedobacter</i>	CMRootsF	Bioccontrol activity, produces siderophores, IAA, protease, chitinase, cellulose, and pectinase.	[8, 108, 109]
Plant Growth Promoter	<i>Enterobacter</i>	CMRootsF	Enhances plant growth through multiple growth-promoting activities.	[91, 92]
Plant Growth Promoter	<i>Ferrovibrio</i>	CMRhizoA, CMRootsA	Proven to have a positive impact on plant growth and considered as PGPB through nitrogen fixation.	[10]
Plant Growth Promoter	<i>Geomonas</i>	CMRhizoA, CMRootsA	Known for nitrogen fixation, an important trait for plant growth-promoting rhizobacteria.	[110]
Plant Growth Promoter	<i>Lelliottia</i>	CMRootsF	Exhibits plant growth-promoting effects through phosphorus solubilization.	[111]
Plant Growth Promoter	<i>Luteimonas</i>	CMRootsF	Endophytic isolates increase nitrogen acquisition, suggesting an important role in plant growth promotion and nitrogen metabolism.	[112, 113, 10]
Stress Mitigate	<i>Agrobacterium</i>	CMRootsF	Infects plant cells and transfers DNA, causative agent of crown gall disease.	“Agrobacterium tumefaciens - an overview — ScienceDirect Topics” (n.d.)
Stress Mitigate	<i>Variovorax</i>	CMRootsF	Promotes plant growth in drying soil conditions.	[10, 114]
Unknown Impact	<i>Tuberibacillus</i>	CMRootsF	Observed in some studies but no detailed characterization provided.	[115]

The study demonstrates that biostimulants derived from *L. indica* culture media significantly influence the rhizosphere and endosphere microbiota, particularly under CM N⁻ conditions at T35. Key genera have been highlighted and prove their roles in promoting plant growth and stress resilience. Biostimulation brings up distinct microbial patterns, with several genera previously associated with *S. lycopersicum* microbiota and flowering stages. These results underscore the potential of *L. indica*-based biostimulants to enhance crop productivity by modulating beneficial microbial communities.

2.4 Relation between phenotypic observation and genomic results on biostimulant impact

The integration of phenotypic and taxonomic results highlights a clear link between biostimulant-induced microbiota modulation and plant growth responses in *S. lycopersicum*. The observed phenotypic differences in plant height and flowers count under various biostimulant treatments align with the shifts in microbial community composition and diversity identified revealing the occurrence of interesting PGPB [116, 117].

Phenotypically, the use of nitrate-deficient culture of *L. indica* (BME N⁻ or CM N⁻) as a biostimulant through amendment application significantly enhanced respectively plant height, and flower production, at T35 compared to the water control, while foliar application of culture media of *L. indica* containing nitrate (CM N⁺) increased plant size. Nevertheless, biostimulants containing nitrate (CM N⁺) presented an inhibitory effect on plant size. These results suggest that the composition of the biostimulant influences its efficacy, notably through its impact on the microbiota associated with plant roots and rhizosphere. Genomic data corroborate these findings, revealing significant shifts in microbial community composition and diversity over time. The α -diversity analysis (Shannon index) demonstrated a notable increase in microbial diversity from T0 to T35 under CM N⁻ and BME N⁻ treatments, suggesting enhanced microbial community complexity. This increase in diversity is consistent with the presence of unique genera, particularly under nitrate-deficient conditions, as evidenced by the Venn diagram analysis (Supplementary Figure 4). PERMANOVA (Supplementary Table 1) further confirmed the significant influence of both time and biostimulant conditions on microbial community structure, supporting the temporal nature of the phenotypic responses.

In addition, key microbial genera enriched under biostimulation treatments, such as *Rhodanobacter*, *Devosia*, *Azospirillum*, and *Flavipsychrobacter*, are recognised for their roles in nitrogen fixation, phytohormone production, and stress resilience [10, 69, 71, 70, 73, 74, 118]. More importantly, *Devosia* and *Azospirillum* are associated with improved nitrogen availability and root development, suggesting a better plant development and therefore aligning with the enhanced flower production observed under CM N⁻ biostimulation [10]. Similarly, *Rhodanobacter* and *Flavipsychrobacter* contribute to auxin production and nutrient uptake, which may explain the increased plant height under BME N⁺ treatments [69, 71, 70, 118]. These genera, enriched predominantly at T35, likely play a role in supporting the later stages of plant growth and flowering as observed by [10] and [55].

Temporal dynamics of microbial community composition further elucidate the relationship between plant phenotypes and microbiota. Early-stage dominance of genera such as *Massilia* and *Rhodanobacter* at T0-T20 suggests their role in plant establishment and early growth [78, 79, 80]. By T35, the enrichment of genera like *Devosia* and *Azospirillum* corresponds with the onset of flowering and biomass accumulation [10, 55]. These temporal patterns reinforce the importance of microbiota establishment over time by benefiting from the biostimulation with *L. indica*.

The demonstrated efficacy of nitrate-deficient *L. indica* cultures (CM N⁻, BME N⁻) as biostimulants emphasises the necessity of optimising formulations to selectively enrich plant growth-promoting microbial genera. Notably, biostimulants derived from CM N⁻ cultures are rich in polysaccharides, as observed in Section 2.1. These polysaccharides have been reported to play a major role in promoting beneficial interactions between plants and microorganisms [119, 120]. Indeed, polysaccharides serve as a crucial energy source for bacteria, facilitating their proliferation and activity, and also contribute to antifungal and pathogen-suppressing effects in plants [121]. Our data highlight clear nitrate-dependent shifts in both internal and excreted polysaccharide profiles. While these observations strongly suggest a potential mechanistic role of polysaccharides in shaping plant-microbiome interactions, we acknowledge that this linkage remains hypothesis-generating, as direct functional assays were beyond the scope of the present study. Nonetheless, these findings provide a solid foundation for future work aimed at experimentally testing the contribution of specific polysaccharide

fractions to plant responses and microbiota modulation. Furthermore, microbial activity associated with these biostimulants has been suggested to enhance nutrient availability and uptake in plants, thereby promoting growth [122, 123]. Additionally, polysaccharides have been linked to improvements in soil structure by increasing soil aggregation and water retention, fostering a more conducive environment for root development [124]. The presence of polysaccharides in biostimulants can also stimulate the production of exopolysaccharides by beneficial bacteria, further reinforcing plant-microbe interactions and enhancing plant resilience to environmental stresses. Altogether, this enriched environment with polysaccharides shows promising properties to prevent and cope with different abiotic stresses [118, 10, 125, 126, 9].

Nevertheless, while polysaccharides play a significant role, other bioactive compounds and free metabolites present in the *L. indica* culture fraction may also contribute to plant-microbe interactions and stress mitigation. Metabolites such as organic acids, volatile organic compounds, amino acids, and phenolic compounds can influence microbial community composition by selectively promoting the growth of beneficial microorganisms or inhibiting pathogens [127, 128, 129, 130, 3]. Organic acids, for instance, are known to facilitate nutrient solubilisation, improving the bioavailability of essential elements such as phosphorus and iron [131]. Additionally, amino acids, vitamins and volatile organic compounds may serve as signalling molecules, modulating microbial activity and inducing plant defence responses [132, 133, 134, 135, 136]. Certain secondary metabolites, including flavonoids and phytohormone-like compounds, could further enhance plant resilience to abiotic stresses by regulating oxidative stress responses and improving water-use efficiency.

Altogether, while polysaccharides represent a key component of these biostimulants, the combined action of multiple metabolites likely contributes to their overall efficacy. Future studies should aim to understand the relative contributions of these various compounds to optimise biostimulant formulations for enhanced plant growth and stress resilience.

3 Discussion

Most studies investigating the biostimulant potential of cyanobacteria have essentially focused on biomass or on specific biomass fractions, such as polysaccharides, proteins, or lipid extracts. By contrast, very few studies have examined the potential role of the residual culture medium, which can accumulate a variety of extracellular metabolites secreted during growth [137]. The downstream management of this culture medium represents a critical bottleneck in the economics of cyanobacteria cultivation, as large volumes are generated and their disposal or recycling impacts production costs. Several studies have therefore considered either the reuse of culture media for successive batches [138, 139] or the substitution of synthetic media with wastewater streams as a more sustainable input [140, 139]. In this context, our work provides novel insights by evaluating, for the first time, the biostimulant potential of the residual culture medium from *L. indica* cultures, particularly under conditions of nitrogen deprivation.

Furthermore, to our knowledge, there are no or very few studies that have addressed this re-

search by modifying the metabolism of *L. indica* during cultivation in such a way as to induce metabolic shifts that cause a drastic change of intracellular and extracellular metabolites. In the present study, we demonstrate that both the biomass and residual culture medium (CM N⁻, BME N⁻) of *L. indica* cultivated under nitrogen deficiency show markedly higher biostimulant activity than when it is grown under nitrate-replete conditions. Nitrogen deprivation induced a substantial increase in polysaccharide synthesis, including extracellular polymeric substances (EPS), and altered the protein-to-polysaccharide ratio. Such shifts are consistent with previous reports in other cyanobacteria and microalgae, where nitrogen stress promotes carbon flux redistribution toward carbohydrate storage and EPS production [141].

Despite the relatively small number of plants tested per treatment ($n = 5$), we observed a significant increase in floral production (+49.8% compared to the water control; p -value <0.05, Student's t-test) in plants treated with the CM N⁻ fraction. These promising results highlight the potential of residual culture medium as a low-cost, sustainable biostimulant. Future studies should aim to increase replication, extend the experimental period, and assess fruit yield and quality. Indeed, microalgal or cyanobacteria treatments, whether in the form of aqueous or ethanolic extracts, crude biomass, polysaccharide fractions, or foliar suspensions, have already been shown to modulate tomato fruit composition, including pigments (carotenoids, lycopene), antioxidants (phenolics), vitamins (*e.g.*, vitamin C), mineral accumulation (Fe, Zn), and sugars [142, 143, 144]. These results strongly suggest that extending the evaluation beyond floral induction to fruit biochemical profiling would provide a more comprehensive picture of the benefits of *L. indica* extracts.

Another key finding of our study concerns the plant-associated microbiome. Interestingly we showed that the profiling of the rhizosphere and endosphere bacteriome revealed a significant change in alpha diversity, particularly with CM N⁻ and BME N⁻ in both applications. The enrichment in polysaccharides under N⁻ conditions appear to enhance specific plant-microbiota interactions, as evidenced in this study. We observed an increase in plant growth-promoting bacteria (PGPB), including *Acidovorax* (increased by 4% from T0 to T35 through soil amendment), *Devosia* (increased by 3% from T0 to T25 through soil amendment, and 4% through foliar application), and *Massalia* (increased by 22% from T0 to T35 through soil amendment), following biostimulation with the CM N⁻ fraction.

These data suggest a direct and an indirect action of the bioactive compounds depending on the mode of application (soil amendment *versus* foliar application) and the extract tested (residual culture media *versus* biomass extract from *L. indica* cultures in presence or nitrate deficient condition). Polysaccharides, particularly those present in residual culture media, are probably involved in the effects observed, both at the phenotypic level and in the microbial level associated to the rhizosphere and endosphere. Indeed, the polysaccharides have already reported to play a major role in promoting beneficial interactions between plants and microorganisms [120, 119]. Polysaccharides serve as a crucial energy source for bacteria, facilitating their proliferation and activity, and contribute to antifungal and pathogen-suppressing effects in plants [121]. Furthermore, microbial activity associated with these biostimulants has been suggested to enhance nutrient availability and uptake

in plants thereby promoting growth [122, 123]. Additionally, polysaccharides have been linked to improvements in soil structure by increasing soil aggregation and water retention, fostering a more conducive environment for root development [124]. The presence of polysaccharides in biostimulants can also stimulate the production of exopolysaccharides by beneficial bacteria, further reinforcing plant-microbe interactions and enhancing plant resilience to environmental stresses. Altogether, this enriched environment with polysaccharides shows promising properties to prevent and cope with different abiotic stresses [10, 125, 118, 9, 126].

Beyond their nutritive role, polysaccharides also function as elicitors of plant defence responses [145]. Compounds such as laminarin, alginates, fucoidans, and carrageenans have been shown to activate defence signalling pathways, modulate secondary metabolism, and alter root exudation profiles, thereby reshaping soil microbial communities [146, 147]. These dual effects, direct metabolic priming of plants and indirect modulation of the microbiome, suggest that polysaccharides are central mediators of the biostimulant activity observed in our study. As demonstrated by [145], ethanol-precipitated purified extracts of polysaccharides from Cyanobacteria, including *L. indica*, show effects on defence and metabolic markers in tomatoes.

Nevertheless, polysaccharides are unlikely to act alone. Cyanobacteria culture media contain a wide array of metabolites, including organic acids, amino acids, phenolic compounds, volatile organic compounds (VOCs), and phytohormone-like molecules, all of which can influence plant physiology and microbiome composition. Organic acids are well-documented for their roles in nutrient solubilization, particularly phosphorus and iron [131]. Amino acids, vitamins, and VOCs can act as signalling molecules that modulate microbial activity and induce systemic plant responses [136, 132, 135]. Secondary metabolites such as flavonoids or indole-like compounds may further enhance abiotic stress resilience by regulating oxidative balance and water-use efficiency [3]. Thus, the combined action of multiple metabolite classes, rather than polysaccharides alone, likely underpins the biostimulant efficacy of the CM N⁻ fraction.

Taken together, our findings support a model in which *L. indica* extracts act through both direct effects on plant physiology and indirect effects mediated via microbiome modulation. Future research should aim to disentangle the relative contributions of these compound classes using fractionation approaches (*e.g.*, purified EPS versus low-molecular-weight metabolites). Integrative approaches combining metabolomics of exudates, plant proteo-transcriptomics, and meta-omics of associated microbiota (metaproteomics, metagenomics, metatranscriptomics) will be essential to elucidate the complex tripartite interactions between plant, bacteria-derived compounds, and the microbiome. In parallel, functional assays (*e.g.*, *nifH* qPCR, enzyme activities) would be beneficial to directly test the functions associated with genera identified in the taxonomic analysis. Additionally, stable isotope tracing (¹³C, ¹⁵N) could be used to follow the transfer of bacteria-derived carbon and nitrogen into plant tissues and microbial communities. Finally, time-course experiments across hours, days, and full crop cycles would provide valuable insights into the persistence and dynamics of these interactions.

In conclusion, this study highlights that cyanobacteria biostimulants are not limited to biomass but also include the residual culture medium. Exploring this often-overlooked component reveals significant biostimulant potential, particularly when cyanobacteria are cultivated under nitrogen stress, a condition that redirects metabolic fluxes toward enhanced production of polysaccharides including EPS. These compounds act not only directly on plant physiology but also indirectly by reshaping the associated microbiome, thereby strengthening plant growth and resilience.

The demonstration that polysaccharide-enriched residual culture medium stimulates flowering and promotes beneficial interactions within the rhizosphere opens new perspectives for sustainable agriculture. By valorising a fraction often regarded as waste, this approach offers a dual advantage: reducing costs linked to spent medium management while increasing the profitability of cyanobacteria production. Thus, the use of residual culture medium as a biostimulant emerges as both an economically attractive and environmentally sustainable strategy, enhancing the overall appeal of *Spirulina* cultivation.

4 Methods

4.1 *Limnospira indica* culture

Limnospira indica strain (PCC8005 from Paster Institute) was maintained in 250 mL Erlenmeyer flasks in photosynthetic growth chamber (MLR-352H-PE, Panasonic, Japan) at 30 °C, 140 rpm shaking and continuous illumination of 60 $\mu\text{mol.photons.m}^{-2}.\text{s}^{-1}$ (Li-193SA; Li-Cor BioSciences, USA). Two media were used: Cogne Modified Zarrouk medium ([148]) (N^+ hereafter) and Cogne Modified Zarrouk medium nitrate depleted (N^- hereafter). Volume was increased in 2 liters glass bottles for biostimulant extracts preparation.

4.2 Biostimulant solutions preparation

Four biostimulant fraction containing potential bioactive compounds from *L. indica* were extracted following the procedure (Figure 10):

- Culture media from a fresh culture of *L. indica* ($\text{OD} = 1,7$) grown in N^+ and N^- conditions was retrieved by centrifugation (3800 rpm for 20 min) after a 20min sonication bath (Elmasonic S 100H) in order to break the vacuoles and enable efficient centrifugation.
- Biomass from a fresh culture of *L. indica* ($\text{OD} = 1,7$) growth in N^+ and N^- conditions was retrieved by centrifugation (3800 rpm for 20 min) after a 20 min sonication bath. The biomass was resuspended in the same volume of Milli-Q water as the initial culture volume and boiled for one hour. The supernatant is retrieved by centrifugation (3800 rpm for 20 min).

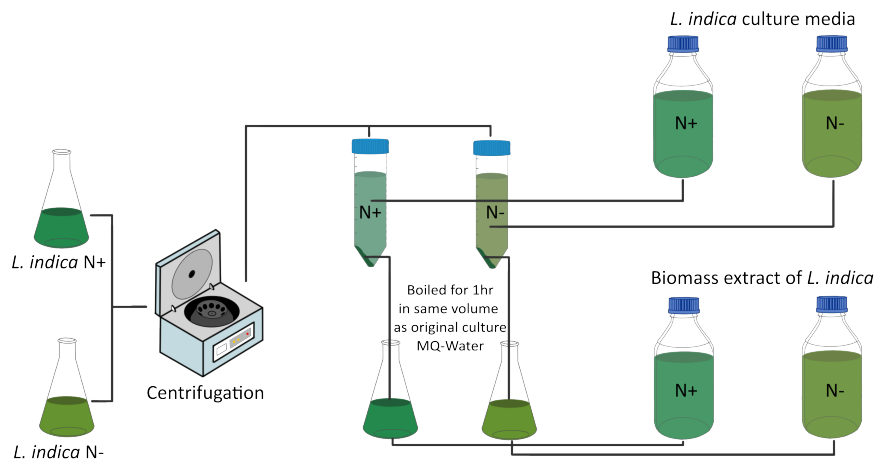


Figure 10: Preparation methods of biostimulant extracts from *L. indica* cultures. *L. indica* cultures (N⁺ and N⁻) are centrifuged, the supernatant is retained for culture media solutions (CM), and the biomass is further processed by boiling to produce the biomass extract (BME). Realised with Procreate[®]

Both N⁺ and N⁻ were harvested together at OD=1,7 (corresponding to a DW of 1.4 in N⁺ condition and 1.6 in N⁻ condition, Supplementary Figure 8) before N⁻ culture start the plateau phase and declined. At this stage, both culture growth were similar (Figure 1). An independent culture per condition has been done before each experiment, described in subsection 4.5. Biostimulant solutions are used immediately after preparation, and are stored at 4 °C for the course of the experiment.

These solutions were diluted in Milli-Q water as following:

- For amendment treatment: culture supernatant solution (2,5% (v/v)), biomass solution (2,5% (v/v)) or water only, with Tween-20[®] (0.01% (v/v)) in all solutions.
- For foliar treatment: culture supernatant solution, biomass solution (25% (v/v)) or water only, with Tween-20[®] (0.01% (v/v)) in all solutions.

Due to volume application constraints on the leaves (leaves area without applications on the soil), biostimulant solution has been concentrated (10X) resulting to a 2.5% concentration when applied by amendment against 25% concentration when applied by foliar application. The pH of the working solutions (mean $\pm$ SD) were: CM N⁺, 10.26 ($\pm$ 0.05); CM N⁻, 10.51 ($\pm$ 0.1); BME N⁺, 8.85 ($\pm$ 0.03); BME N⁻ 8.74 ($\pm$ 0.02) Negative control 9.84 ($\pm$ 0.02). In addition, Tween-20[®], as a surfactant, was used to increase the cells permeability, allowing biostimulant compounds to penetrate the plants tissues ([149, 150]).

4.3 *L. indica* culture fraction characterisation

For carbohydrate quantification, *L. indica* culture media (supernatant) were first washed on ultrafiltration units (Vivaspin[®] 100 KDa, Sartorius[®]) to eliminate salts present in the medium and concentrate exopolysaccharides in MQ-water. The concentrated supernatants were mixed with 3 volumes of absolute ethanol to precipitate polysaccharides. In parallel, *L. indica* cells were freeze-dried (FINN-AQUA[®], LYOVAC GT 2E) before being incubated for 2 hrs at 100 °C in HCl 0.6 M to collect capsular and internal polysaccharides.

Carbohydrate assay was performed using ([151]) methods as follows. 10 μL of phenol 80% (80:20 v/v in ethanol) solution and 1 mL of 98% H_2SO_4 were respectively added to 400 μL of samples. A standard curve was generated with D-glucose as follows: 0 $\mu\text{g.mL}^{-1}$, 12.5 $\mu\text{g.mL}^{-1}$, 25 $\mu\text{g.mL}^{-1}$, 50 $\mu\text{g.mL}^{-1}$, 100 $\mu\text{g.mL}^{-1}$, 150 $\mu\text{g.mL}^{-1}$. After 10 minutes of incubation at room temperature, the optical density was read at 490 nm.

Safety hazards: **Phenol** may cause damage to organs through prolonged or repeated exposure. Phenol is a mutagen and may cause damage to organs through prolonged or repeated exposure. **Sulfuric acid, H_2SO_4** may irritate and burn the skin and eyes, and may lead to blindness. Exposures may cause headache, nausea and vomiting.

For Protein quantification, *L. indica* biomass was filtered on 0.2 μm (Sartorius[®], cellulose nitrate filter) using a vacuum pump. *L. indica* biomass was freeze-dried (FINN-AQUA[®], LYOVAC GT 2E). Dried samples were resuspended in a lysis extraction buffer from Preomics iST Sample preparation kit[®]. Samples were vortexed at room temperature for 20 min followed by a 10 min incubation at 95 °C at 1000 rpm. Then, a sonication by probe step (3x10 sec, amplitude 20%, IKA U50 sonicator) at 4 °C was performed. Cell debris were removed by centrifugation (15 min, 1100 rpm, 4°C). Proteins were recovered within the supernatant. Protein assays were performed using Bradford method ([152]). The standard curve was done by using BgG in MQ-water as follows: 0 $\mu\text{g.}\mu\text{L}^{-1}$, 1 $\mu\text{g.}\mu\text{L}^{-1}$, 2 $\mu\text{g.}\mu\text{L}^{-1}$, 5 $\mu\text{g.}\mu\text{L}^{-1}$, 8 $\mu\text{g.}\mu\text{L}^{-1}$, 10 $\mu\text{g.}\mu\text{L}^{-1}$. Colorimetric assay was performed using bradford reagent, the optical density was read at 595 nm, immediately after reagent mix.

Last, nitrate assays were performed following [153] protocol. 125 μL of sample were mixed thoroughly with 800 μL of 5% (w/v) salicylic acid in concentrated H_2SO_4 . After 20 minutes of incubation at room temperature, 9.5 mL 2N NaOH were added slowly before cooling at room temperature. The standard curve was generated with NaNO_3 as follows: 20 mM, 10 mM, 2.5 mM, 1.25 mM, 0.6 mM, 0.3 mM, and 0.15 mM. Samples and standards were read within 20 min at 410 nm.

Safety hazards: **Sulfuric acid, H_2SO_4** may irritate and burn the skin and eyes, and may lead to blindness. Exposures may cause headache, nausea and vomiting. **Sodium hydroxide, NaOH** may causes severe skin burns and eye damage.

4.4 Composition of Polysaccharides from *Limnospira indica*

Polysaccharides from supernatant (released polysaccharides, RPS) and biomass (capsular and endopolysaccharides) of both nitrate-deficient (N^-) and nitrate-rich (N^+) cultures composition in monosaccharides was analysed. Polysaccharides from the supernatant were concentrated using ultrafiltration units (Vivaspin[®] 100 KDa, Sartorius[®]) (centrifugation at 4000 g for 10 min) before being washed 3x with MQ-water. In parallel, *L. indica* cells were freeze-dried (FINN-AQUA[®], LYOVAC GT 2E). The following saccharides were used as standard: D-Glucuronic acid, D-Glucose, D-Mannose, D-Xylose, D-Galactose, D-Arabinose, L-Rhamnose, D-Galacturonic acid, D-Ribose, Fucose, N-Acetylglucosamine, N-Acetyl Galacturonic. All saccharides are diluted in ULC-MS water at a concentration of 50 mg/mL.

Concentrated RPS, dried biomass and 20 μL of standards solutions were hydrolysed with 2 mL of 4 M TFA (Trifluoroacetic acid) at 98 °C for 5 hours. The samples were evaporated to dryness and

the pellets were resuspended in 500 μ L of ULC MS water. Samples were filtered through a 0.2 μ m membrane (Whatman[®] Mini-UniPrep[®]).

Liquid chromatography was performed using a Sciex Exion LC HPLC. All samples were separated on a XBridge[®] BEH Amide column (130 Å, 2.1 $\times$ 150 mm, 5 μ m) (Waters Corporation[®], 186006590). The solvent system consisted of two mobile phases as follows: mobile phase A (H₂O, formic acid 0.1%) and mobile phase B (Acetonitrile 99.9%, formic acid 0.1%). The column operated at a flow rate of 0.2 mL.min⁻¹ at 40 °C. The gradient elution program was as follows: 0–1 min, 15% B; 1–5 min, 15–17% B; 5–9 min, 17–20% B; 9–12 min, 20–25% B; 12–12.5 min, 25–35% B; 12.5–13.5 min, 35% B; 13.5–14 min, 35–15% B; 14–15 min, 15% B.

Mass spectrometry was performed on a high-resolution mass spectrometer (SCIEX ZenoTof[™]7600) acquiring data in full scan ion mode and tandem MS/MS using an electrospray ionisation (ESI) source. Fragmentation was performed using collision-induced dissociation (CID). MS was operated using a positive mode (collision energy voltage 10 V, electrospray voltage 5500 V, declustering potential 50 V) with a source temperature of 200 °C. MSMS was performed using a negative mode as well (collision energy voltage 40 V, declustering potential 50 V, CE spread 20 V).

Analysis were performed using SCIEX OS software (version 3.3.1.43).

Safety hazards: **Trifluoroacetic acid, TFA** is a corrosive chemical that may severely irritate and burn the skin and eyes with possible eye damage. Breathing Trifluoroacetic Acid may irritate the nose, throat and lungs.

4.5 *Solanum lycopersicum* seedling growth biostimulation

The GreenHab environment was prepared to suit the needs of *S. lycopersicum* of two-leaf stage for 5 weeks. The experiment was conducted in a greenhouse (Mammoth PRO + DR 240 L) under controlled conditions with a 16h light/8h darkness cycle and an average temperature over time of 26 °C. Two lights General Electric 400 W XO HPS were used for the illumination. The temperature and the air flow inside the GreenHab were regulated by one air extractor (VK 200U 780 m³/h) and by 3 fans continuously turned ON. The light was switched ON from 7 a.m. to 8 p.m. each day, and the intensity during the day was 440 μ mol.photons.m⁻².s⁻¹.

The tomato plants *Solanum lycopersicum* (variety MoneyMakers) (ordered at the C.A.R.A.H, Ath, Belgium) were received at a 2-leaves stage, 3 weeks after being sown, n=5. The sample size was defined to satisfy statistical validations while fitting our GreenHab volume constraints. The plants were grown in the greenhab for 2 days before being transferred to fresh soil (DCM Potground for Professionals, pH = 6) in 10 cm diameter pots. Pots were circular (top $\varnothing$ 8.5 cm, bottom $\varnothing$ 7.5 cm, height 7.0 cm) with an internal geometric volume of $\approx$ 0.352 L. All plants were potted using soil from the same batch. Plants were watered with Milli-Q water for 7 days before the experiment, every 2 days for amendment application and every day for foliar treatment. Foliar treatments were prepared at a tenfold higher concentration than the soil amendment, minimising application volume and improving leaf uptake while preventing runoff.

For amendment treatment, biostimulant solutions were administrated (100 mL of 2.5% solution for the 19 first days, then 200 mL of 2.5% solution for the following 15 days) on the soil by carefully

avoiding any contact with the leaves (Figure 11).

For foliar treatment, biostimulant solutions were administrated by spraying them onto the leaves every day (5 mL of 25% solution for the first 19 days, then 10 mL of 25% solution for the following 15 days) by carefully avoiding the soil (soil was shielded using a pot-sized cardboard cover) while Milli-Q water (100 mL for the 19 first days, then 200 mL) was applied on the soil (Figure 11).

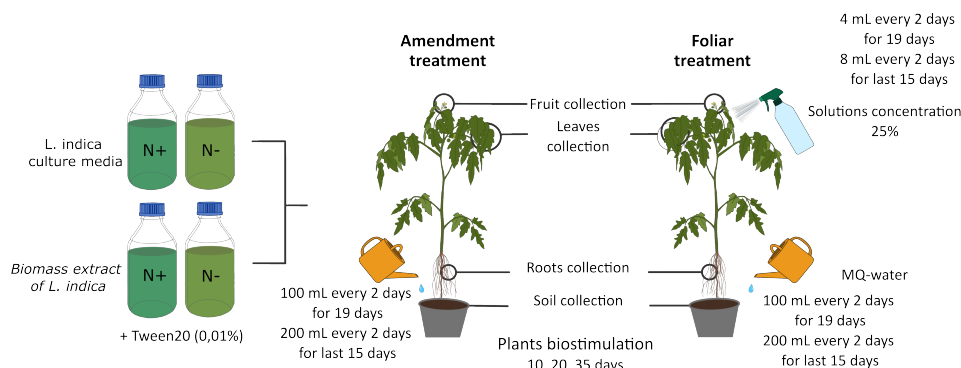


Figure 11: Methods of application of biostimulant solutions extracts during amendment and foliar treatment. Biostimulant were applied on the leaves or the soil and samples were collected after 0, 10, 20 and 35 days. Realised with Procreate®

Biostimulant solution for amendment treatment and watering solution for foliar treatment was top-applied to the soil surface within a $\approx 2-3$ cm radius of the stem, solutions were not mixed into the substrate. Pots sat on saucers and were inspected after dosing; no leachate was observed, indicating full retention in the substrate. The irrigation followed a standard greenhouse regime applied equally across treatments.

Plants characteristics (size of the plant, number of branches, leaves, floral buds, flowers, and early fruits) are collected after 0, 10, 20 and 35 days, after transplanting, reaching stage 6 of the BBCH scale ([154], Supplementary Figure 9). This time point was selected as it corresponds to the onset of the reproductive stage in tomato, a phenological important transition where biostimulants may exert significant effects. Here, because measurements were taken at 35 days, the flower stage and fruits production responses capture early reproductive phenology (flowering onset/initial fruit set). In parallel, the roots and the rhizosphere soil are collected for bacterial sequencing analysis (16S sequencing). Due to space management, the samples have been divided in 3 batch experiments (following each other in time). NMDS and PERMANOVA analysis has been performed on T0 from each batch in order to determine our ability to directly compare batches together (Supplementary Figure 10, Supplementary Table 5). Our analysis revealed that T0 were different and for full transparency, batches analysis are kept separate. Samples were further analysed by experiment, represented in the following table:

Table 3: Sample conditions analysed in this study and associated batch experiments.

Batch	Abbreviation in text	Biostimulant type	Biostimulant application	Sample type
1	CMRhizoA	Culture Media (CM)	Amendment	Rhizo
1	CMRootsA	Culture Media (CM)	Amendment	Roots
2	CMRhizoF	Culture Media (CM)	Foliar	Rhizo
2	CMRootsF	Culture Media (CM)	Foliar	Roots
3	BMRhizoA	Biomass Extract (BME)	Amendment	Rhizo
3	BMRootsA	Biomass Extract (BME)	Amendment	Roots
3	BMRhizoF	Biomass Extract (BME)	Foliar	Rhizo
3	BMRootsF	Biomass Extract (BME)	Foliar	Roots

4.6 Biostimulant impact on rhizosphere and endosphere microbiota

In this study, the rhizosphere denotes soil present around the root system adhering to the root system (root-associated soil, centre of the bulk soil). The root fraction comprises microorganisms associated with the roots, including both surface-attached (rhizoplane) and internal (endophytic) communities. To capture both compartments, we intentionally did not perform surface sterilisation. Accordingly, this fraction represents the combined root-associated community rather than a strictly sterilised endosphere.

DNA extraction, amplification and 16S rRNA amplicon sequencing were performed as following: Samples (Table 3) of the rhizosphere soil and roots were collected in sterile conditions (under flame) for targeted genomic analysis after 0, 20 and 35 days. Roots were washed, in sterile conditions, in 3 sterile PBS baths to remove the remaining soil. 2 mL PBS was added to the primarily cut off roots before crushing them using the UltraTurax. Samples were centrifuged at max speed for 15 min, supernatant is kept at -40 °C. DNeasy Power Soil kit (Qiagen®) was performed using the pellet for the roots sample and 250 mg of soil (without remaining roots) for the rhizosphere samples, following the manufacturer’s instructions. Subsequent DNA was quantified using the QuantiFluor® ONE dsDNA System (Promega®).

To characterise the bacterial communities, the entire 16S rRNA gene was amplified using the 16S Barcoding Kit 24 V14 (SQK-16S114.24, Oxford Nanopore Technologies) allowing for broad taxonomic resolution. The DNA was amplified by PCR using specific 16S primers ([155]):

27F (5'-AGAGTTTGATCMTGGCTCAG-3')

and

1492R (5'-CGGTTACCTTGTTACGACTT-3'),

which spans nearly the full length of the 16S rRNA gene ([156]) and contains 5’ tags which facilitate the ligase-free attachment of Rapid Sequencing Adapters added in the library. DNA amplification was performed using 20 ng of DNA. The PCR conditions were set according to the kit’s protocol, with an initial denaturation of 1 min at 95 °C followed by 25 cycles of denaturation (20 secs at 95 °C), annealing (30 secs at 55 °C) and extension (2 mins at 65°C), and a final extension 5 mins at 65 °C. Barcodes were ligated to the amplicons according to the kit’s protocol during the

PCR, allowing multiplexing of samples. 80 ng of amplified DNA were purified using the manufacturer’s instructions and DNA quantification is performed using the QuantiFluor® ONE dsDNA System (Promega®). Purified 16S rRNA gene amplicons were prepared for sequencing using the 16S Barcoding Kit SQK-16S114.24.

The sequencing library was prepared by following the manufacturer’s instructions using 50 ng of purified DNA combined from 24 samples per flow-cell. The sequencing was carried out on the GridION Mk1 using flow cells (FLO-MIN114.8) with a pore count of above 1200. The sequencing run was conducted for 20 hours with a minimum read length of 1000 bp, a high-accuracy basecalling, a sequencing score of 9 and a minimum barcoding score of 60.

The taxonomic identification was performed with the EPI2ME tool (Oxford Nanopore), workflow wf-16s for the identification of the origin of single reads from 16S amplicon sequencing. The basecalling and demultiplexing were done using MinKNOW (24.02.16) and Dorado (7.3.11, integrated in MinKNOW), as well as the workflow WF_16S v1.5.0, which uses minimap2 (v2.26-r1175). The following parameters has been used: Classification method = minimap2, Database = ncbi-16S-18S, taxonomic rank = G, Minimum reads length = 1200, Maximum reads length = 1800, Minimum score quality = 9. After identification, all identifications with less than 10 reads or identified as "incertae sedis" ([157]) are removed from the analysis.

Quality of the sequencing has been verified through rarefaction curves analysis (Supplementary Figure 11, Supplementary Table 6) using a Python script (in Supplementary Script 1) ([158]). The α -diversity (Shannon index) was calculated on each rarefied sample set (Limma RGui package, using RStudio version 2024.04.2), excluding sample with less than 40,000 reads for rhizosphere samples and less than 4,000 for endosphere samples, resulting to the exclusion of one unique sample (labelled in red in Supplementary Table 7). The β -diversity was first assessed through non-metric MultiDimensional Scaling (NMDS) profile of the pairwise community dissimilarity (Bray-Curtis) indices of the 16S sequencing data was realised using the PAST software (version 4.17) ([159, 160]). Venn diagrams were produced using Rgui on RStudio ([161, 158]) to determine the genus distributions of different conditions. β -diversity was evaluated using dissimilarity and multivariate analyses by using the Rgui package vegan (version 2.6-8) using RStudio ([162]). Relative abundance is represented in the present study using Circos online tool (<https://mk.bcgsc.ca/tableviewer/visualize/>) allowing highlight of similarities and differences from the 10 most representative genus per condition ([88]). Last, to test the significance of the factors analysed (Condition, Time), PERMANOVA tests were performed on the Bray-Curtis dissimilarity using 100,000 permutations using RStudio. In addition, PERMANOVA with the strata argument was applied to account for inter-batch variation, and pairwise comparisons between conditions were assessed using the pairwiseAdonis method.

The identification and validation of Response Groups (RG) were performed following [158] analysis method. The total of genus identified amongst the 8 datasets analysed in the study (Table 3) as significantly affected genus by the condition are plotted on respective heatmaps. These genus were defined by the deviance analysis on the rarefied counts under nbGLM (negative binomial distribution and Generalized Linear Model) revised by 1000 resampling iterations of the residual variance

by the utilisation of the Rgui package mvabund ([163]). RGs were defined with a hierarchical cluster dendrogram (Euclidean distance and average clustering) using the Rgui package vegan ([162]). The statistical validity of these groups was assessed by comparing the RG clustering with a null-model by Monte-Carlo simulation (Supplementary Figure 12) by using all the genus to reinforce the randomisation process.

4.7 Data availability

All raw data are available in NCBI repository, under project accession PRJNA1328183: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1328183>

4.8 Code availability

Python code used for rarefaction is provided in Supplementary data.

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Authorship contribution statement

CR: Methodology (General), Experimentation (General), Investigation (Data visualisation, Data validation, Statistical analysis, Formal analysis), Writing (original draft, review and editing). AD: Investigation (Data visualisation, Data validation, Statistical analysis), Writing (Review). PL: Experimentation (Sequencing). CM: Methodology (monosaccharides analysis), Experimentation (monosaccharides analysis). RW: Project administration (Funding acquisition, Supervision), Investigation (Data validation, Formal analysis), Writing (Review and editing).

Declaration of competing interest

The authors declare that they have no known competing financial interests or non-financial or personal relationships that could have appeared to influence the work reported in this paper.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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