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ENVIRONMENTAL MICROBIOLOGY - RESEARCH PAPER

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Effect of soil chemical fertilization on the diversity and composition of the tomato endophytic diazotrophic community at different stages of growth

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Abstract

The aim of this work was to gain a more comprehensive and perspicacious view of the endophytic diazotrophic community (EDC) of tomato plant bacteria and assess the effects of chemical fertilization and the plant phenologic stage on the status of those microbes. When the EDC of stem and roots from tomato plants grown in a greenhouse with and without exogenous chemical fertilization was examined by pyrosequencing the *nif*H gene during the growth cycle, a high taxonomic and phylogenetic diversity was observed. The abundant taxa were related to ubiquitous endophytes such as *Rhizobium* or *Burkholderia* but also involved anaerobic members usually restricted to flooded plant tissues, such as *Clostridium*, *Geobacter*, and *Desulfovibrio*. The EDC composition appeared to be dynamic during the growth phase of the tomato, with the structure of the community at the early stages of growth displaying major differences from the late stages. Inorganic fertilization negatively affected the diversity and modified the profile of the predominant components of the EDC in the different growth stages. Populations such as *Burkholderia* and *Geobacter* plus the Cyanobacteria appeared particularly affected by fertilization.

Our work demonstrates an extensive endophytic diazotrophic diversity, suggesting a high potential for nitrogen fixation. The effect of the phenologic stage and inorganic-chemical soil fertilization on the community structure indicated a dynamic community that responded to environmental changes. These findings contribute to a better understanding of endophytic associations that could be helpful in assisting to shape the endomicrobiome that provides essential benefits to crops.

Keywords Endophytes · N_2-fixing bacteria · Tomato · *nif*H gene pyrosequencing

Introduction

Plants play a key role in the creation and promotion of rhizospheric and endophytic environments by releasing exudates with different organic compounds. Endophytic environments refer to specific locations inside plant organs or tissues in which bacteria have been found to have neutral, detrimental, or beneficial effects on the host plant [1]. Endophytes have gained attention for their ability to confer positive effects on plant growth as well as their indirect influence in acting as biocontrol agents. Much of the information about the functions that endophytes could be playing inside plants comes from studies with rhizobacteria, in particular with model plant growth-promoting rhizobacterial strains [2]. Among the beneficial endophytes, diazotrophic bacteria have been isolated from plant species such as rice, sugar cane, and wheat [3–5]. Biological N_2 fixation—the incorporation of molecular N_2 into the biologically available ammonium, here by diazotrophs—accounts for approximately 128 million tons

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nitrogen per year and is considered the main route by which fixed nitrogen enters the biosphere by natural processes [6]. The expected rise in the world population will lead to a higher crop production by agrosystems in which the use of N-fixation by bacteria represents a convenient alternative for profitability and sustainability. Biologic N_2 fixation—it being catalyzed by only bacteria and archaea—requires the enzyme nitrogenase, an evolutionarily conserved protein present in N_2-fixing microorganisms. Nitrogenase is composed of two multisubunit metalloproteins. Component I contains the active site for N_2 reduction and is composed of two heterodimers encoded by the *nif*D and *nif*K genes. Component II, also known as dinitrogenase reductase, contains two identical subunits encoded by the *nif*H gene. The *nif*H gene sequence is highly conserved throughout the bacterial and archaeal domains [7].

The current state of knowledge shows us the endophyte microbiome of plants comprised of a diversity of phylotypes with representatives of all bacterial phyla including Proteobacteria, Actinobacteria, and Firmicutes, as well as new uncultivated lineages discovered in recent years [8–12]. Metagenomic studies have revealed that factors such as plant species, organ type, phenologic stages, soil type, and fertilization affect the endomicrobiome of plants [13]. Nevertheless, determinants of diazotrophic bacterial diversity within the populations inhabiting plants still remain largely unexplored. The diversity of the endophytic diazotrophic community across different perennial grass species is affected by the type of soil, and N fertilization which strongly reduces the activity and abundance of some of these populations [14, 15]. Studies on tropical rice reveal flooding as a major effector of the diversity and composition of the entire endophyte community as well as the N-fixing endophytes [16, 17].

The tomato (*Solanum lycopersicum* L.) is one of the main green vegetables produced in Argentina, especially in greenhouses. The endophytic bacterial community of tomato has been analyzed by culture-dependent approaches and through direct analysis of 16S rRNA sequences [18–21]. In contrast, functional approaches are less numerous and mainly focused on the ability of tomato endophytes to confer resistance to biotic or abiotic stresses [22–24]. To the best of our knowledge, there are no previous metataxonomic studies on the functionality of the tomato endophytic community, particularly on its potential to fix N. The aim of this work was therefore first to acquire an extensive characterization of the endophytic community in tomatoes that is involved in fixing nitrogen and then assess the effects of chemical fertilization and the plant phenologic stage on those populations. We hypothesized that the diazotrophic populations inside tomato plants play an important role in the early growth stages and are differentially affected by fertilization. Our results revealed compositional variations in the community throughout the plant growth period as well as the presence of novel components and evidenced that chemical fertilization adversely affected the diversity as well as the abundance of the predominant diazotrophic taxa.

Materials and methods

Plant growth conditions

Tomato plants (cv. Elpida) were grown in a plot of soil in a greenhouse at the Estación Experimental Julio Hirschhorn in La Plata (Buenos Aires province, Argentina; 34° 59′ 06″ S; 57° 59′ 50″ W). The soil under study was a silty loam Typic Argiudoll [25] of the Bombeador series containing 2.1% organic matter, 0.22% total nitrogen, 6.8 ppm of extractable phosphorus, and a pH of 5.8. The plants were watered by drip irrigation during the experiment; after the transplantation, one group of plants was treated with tap water alone (nonfertilized plants), whereas another was irrigated every 6 days with a solution composed of 150 ppm N and 80 ppm of P and K (fertilized plants), which composition corresponds to that routinely used under the standard conditions of tomato fertilization. The experimental design was completely randomized and consisted of four plots, two replicate for each treatment; each plot was 6.5 m long and 1.5 m wide with 26 plants distributed in two rows. The spacing was 0.7 m between rows, 0.4 m between plants, and 1.2 m between plots. The plants were harvested at different developmental stages—namely, at early vegetative growth, immediately before transplanting (stage 1, M1); after flowering (stage 2, M2); and at senescence (stage 3, M3), respectively 30, 90, and 120 days after germination. Five plants for each replicate plot (ten per treatment) were randomly sampled at each stage and immediately processed for DNA extraction.

DNA extraction from tomato plant endophytes

A destructive procedure for DNA extraction was performed on two composite samples (independent biological replicates), each consisting of roots and stems of 5 pooled plants for each replicate plot. A destructive sampling method for DNA extraction was performed on two composite samples (independent biological replicates), each consisting of the roots and stems of the 5 pooled plants for each replicate plot. First, the tissues were thoroughly washed with tap water, and the rhizoplane microbiome eliminated by washing with 0.2% (w/v) sodium dodecyl sulfate for 15 min with continuous shaking, followed by washing with water and incubation for 15 min in 5% (w/v) sodium hypochlorite. Finally, the plant material was washed in 70% (v/v) aqueous ethanol for 15 min and rinsed three times with sterile distilled water. To examine the surface disinfection efficiency, aliquots of the water used in the last wash as well as pieces of root and stem tissue were each placed in solid tryptone soya agar medium for 1 week at

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28 °C. Subsequently, the plates were examined for the presence of growing microbial colonies. The surface-disinfected tissues were quick frozen in liquid nitrogen and thereafter kept at − 80 °C until used for DNA extraction. Metagenomic DNA was extracted according to the protocol described by Murray and Thompson [26].

Sequence analysis of the *nifH* locus

Amplification of an internal fragment (360 bp) of the *nif*H gene was performed by means of a nested PCR as proposed by Yeager et al. [27] with the slight modifications described by Calderoli et al. [28]. The procedure stated in brief: Of the DNA from the disinfected tissues, 20 ng was amplified through the use of the primers 19F and nifH3. For the second PCR, 2 μl of undiluted PCR product was used as a template and the primers included the Roche-454 tag sequences indicated in bold fused to the 5′ terminus (nifH11, 5′-**CACG ACGTTGTAAAACGAC** GAY CCN AAR GCN GAC TC-3′, and nifH22, 5′-**CAGGAAACAGCTATGACC** ADW GCC ATC ATY TCR CC-3′). The PCR reactions were conducted in triplicate to minimize random PCR bias. The amplicons were purified and quantified with a Nanodrop Spectrophotometer. Technical replicates were pooled in equimolar concentrations in a single library; a total of ten DNA samples were labeled with a unique oligonucleotide bar code and pyrosequenced by means of 454 GSFLX technologies.

The pyrosequencing data were processed as previously described by Collavino et al. [29]. In a brief description, short and low-quality reads, putative frameshifts, and chimeras were removed, with the resultant reads clustered with 98% amino acid sequence similarity through the use of CD-HIT software [30]. Operational taxonomic units (OTUs) with at least three sequences were selected and their relative abundance was normalized according to the subsampling method of Mothur [31]. OTU amino acid sequences along with sequences from the *nif*H reference database (https://wwwzehr.pmc.ucsc.edu/nifH_Database_Public/) were used to build protein phylogenetic trees. The *nif*H sequences have been deposited at NCBI-SRA under the BioProject number PRJNA531278.

Statistical data analysis

The α-diversity of the bacterial endophytic diazotrophic communities (EDCs) was examined through the use of the following indices of diversity and richness and rarefaction curves. We calculated the Chao1 (S_{Chao1}), Shannon (H'), and Simpson (1-D) indices by means of the Past3 program [32]; whereas for the rarefaction curves, we used the rarefaction single Mothur command [31]. To determine the significance of diversity parameters across treatments, analysis of variance coupled with a posteriori Fisher's comparison test was applied with the statistical software InfoStat version 2020 [33]. The lack of homogeneity in variance between treatments for indices Shannon, Chao, and Sobs was corrected according to a mixed linear model using the varIdent function from the Infostat software and its interface with R [34].

The variation in the composition of the communities (ß-diversity) was examined through the application of two measurements of dissimilarity: first, the Bray–Curtis dissimilarity test calculated from the abundance of *nif*H subclusters and second the weighted UniFrac distance matrix applied to the abundance and phylogeny of the *nif*H OTUs. These measurements were calculated with the Past3 program and according to the weighted UniFrac command from the Mothur program, respectively. The principal coordinates analysis (PCoA) was used to visualize the differences between the communities. Statistical differences were analyzed considering the two main factors—fertilization and phenological stage with a permutational multivariate analysis of variance (PERMANOVA) implemented through the function Adonis from the vegan package [35] of R using the Bray–Curtis dissimilarity with 999 permutations.

Results

Negative effect of soil fertilization on the diversity of the EDC

We examined the endophytic community of tomatoes grown in a greenhouse under two experimental conditions—namely with and without exogenous chemical fertilization of plants—by means of a DNA sequencing analysis of the *nif*H locus. The plants were sampled at different times during growth from the plantlet stage through senescence. Harvested roots and stems, after surface sterilization, were pooled for DNA extraction and the DNA then used as a template for the PCR amplification and pyrosequencing of the *nif*H locus. From a total of 70,733 high-quality reads, 10,776 unique protein sequences and 947 OTUs at a 98% similarity were obtained, thus indicating a high level of diversity within the EDC community of tomato tissues.

The diversity of EDC among plant samples was compared by means of rarefaction curves and various estimators of richness and evenness (Fig. 1, Table 1). At a 2% similarity, none of the rarefaction curves reached saturation (Fig. 1), but the relative coverage values were nonetheless found to be similar among the samples, ranging from 50 to 72%, which variation pointed to a substantial degree of diversity in all the samples (Table 1).

The effect of fertilization on EDC diversity varied across treatments. The highest and lowest Shannon and Simpson values were observed respectively in unfertilized and fertilized flowering plants, indicating the negative effect of fertilization

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Table 1 Sample coverage and diversity indices for the *nif*H libraries evaluated in the different phenological stages of tomato without fertilization (M1, M2, and M3, respectively) and fertilized (M2_fert and M3_fert)

Indices	M1	M2	M2_fert	M3	M3_fert
SeqN[a]	14,463	2165	7627	5112	5997
SeqNn[b]	2135	2130	2134	2130	2133
Sobs[c]	102.5a	176.0c	126.0ab	237.5c	168.5bc
H' (Shannon)	1.62a	3.28c	1.58a	2.62b	2.21b
1-D (Simpson)	0.65b	0.92a	0.50c	0.71b	0.67b
D (dominance)	0.35b	0.08a	0.50c	0.30b	0.33b
S_{Chao1} (Chao1)	231.80a	214.65a	253.75ab	399.35c	346.60bc
R_{Chao1}	50	72	50	60	51

[a] SeqN, the number of sequences per sample

[b] SeqNn, the normalized number of sequences per sample

[c] Sobs, the number of operational taxonomic units (OTUs) at 2% distance level

[d] R_{Chao1}, the relative coverage, calculated as the OTU number divided by the estimated richness (S_{Chao1}). All the values correspond to the adjusted means of two biological replicates. Different letters between treatments (columns) indicate statistically significant differences (p value ≤ 0.005)

at this stage, whereas no significant differences were observed by fertilization in senescence (Table 1). The diversity in the vegetative stage was consistently low, similar to that found in fertilized flowering plants (Shannon index) or senescent plants (Simpson index). Conversely, species richness was not affected by fertilization but by the growth stage. The senescent plants evidenced values of the metric indicators—the number of species observed (Sobs) and the Chao index— significantly higher than the vegetative plants (Table 1). Principal component analysis biplots show a more comprehensive view of the relationships between treatments as defined by their EDC diversity values (Fig. 2). The first component (CP1) mainly differentiates the fertilization treatments in the flowering stage, with high diversity values (Shannon and Simpson indices) in nonfertilized plants. In contrast, fertilization at this stage decreases EDC diversity and increases dominance. The second component (CP2) differentiates the senescence stage from the rest of the stages, mainly associated with an increase in EDC richness (Sobs and Chao index) (Fig. 2).

These results demonstrated that at the early stage of growth, tomato plants displayed distinctive endophytic populations, which community then changed during maturation. Probably seed-borne bacteria became dispersed within the emerging plantlets during growth, as had been previously described for several plant species [36]. Subsequently, interactions with the soil bacterial community promoted new

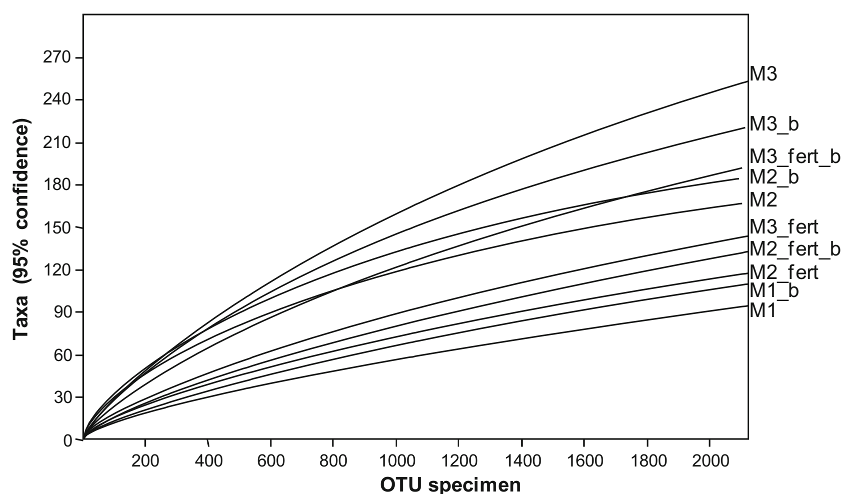

Fig. 1 Rarefaction curves based on operational taxonomic unit (OTU) numbers observed in the endophytic diazotrophic community (EDC) of tomato plants. In the figure, the number of taxa (at 95% confidence interval) recorded is plotted on the *ordinate* as a function of the number of OTU specimens monitored on the *abscissa*. Each curve represents a biological replication with two per sample (−a and −b) of the different phenological stages and without and with fertilization: In this and the other figures, the abbreviations are as follows: M1, plants in the vegetative state; M2, flowering plants; M3, senescent plants—all without fertilization; M2 fertilized, M3 fertilized—those stages with fertilization

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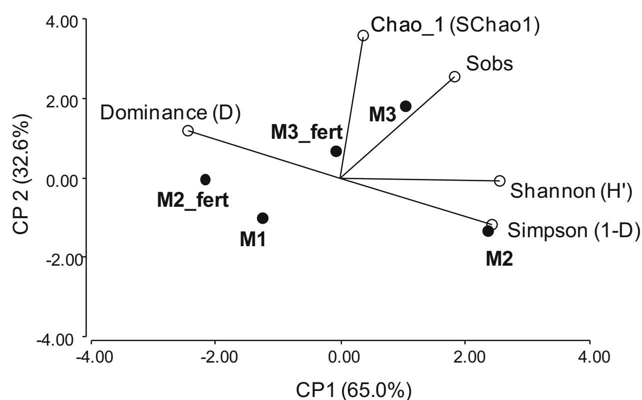

Fig. 2 Biplot from the principal component analysis of EDC diversity in the different phenological stages of tomato plants with and without exogenous chemical fertilization. Treatments (black circles) correspond to the different phenological stages of tomato without fertilization (M1, M2, and M3, respectively) and fertilized (M2_fert and M3_fert). The model used to explain variability included the following parameters (white circles): dominance, Chao1, Sobs, Shannon, and Simpson indices. The first component (PC1) covers 65% and the second component (PC2) an additional 32.6% of the total variation

associations, which populations at the time of senescence extended to a broad spectrum of species. This interaction appeared to be affected by exogenous inorganic fertilization, causing a different extent of decrease in the EDC diversity during the different growth stages.

Effect of soil fertilization on EDC composition at different stages of tomato growth

Sequences of the *nif*H locus were used to identify the genotypes of the EDC according to the cluster grouping and classification proposed by Zehr et al. [7]. Thus, sequences were assigned to 9 out of 17 subclusters comprised in the *nif*H phylogeny with a clear predominance of certain ones (Table 2). The predominance of subcluster 1J/1K, mainly composed of Burkholderiales, Rhizobiales, and uncultivated sequences, ranged between 42 and 95% among the samples. Taxa clustering in 1A (Desulfuromonadales) and 1E (*Paenibacillus*) were also found to be abundant and well represented in all the samples, whereas subclusters 1G and 3, related to Pseudomonadales and Enterobacteriales, and Clostridiales and Desulfovibrionales, respectively, were also represented in the database but at a lower distribution among the samples.

To identify relationships between samples, we examined the variation in the composition of the communities (ß-diversity) by using the principal coordinates analysis based on weighted UniFrac distances and Bray–Curtis dissimilarities. The results of both analyses showed similar sample ordinations and a high percentage of total variance explained in both components (PC1 47.5% and PC2 21.3% for UniFrac, 55.9% and 26.7% for Bray–Curtis). The results for Bray–Curtis ordination are shown in Fig. 3a, whereas not shown for weighted UniFrac analysis. There is a clear separation between M1 and other phenological stages along the *abscissa* in the score plot (PC1). PC1 and PC2 appear to group together the fertilized plants (M2_fert and M3_fert) and stage M3 of unfertilized plants, whereas M1 and M2 group separately. The two-way NPMANOVA test confirmed the significance of the main factors (phenological stage $p = 0.002$, fertilization $p = 0.026$), as well as their interaction (0.035). As a result of fertilization, the flowering plants (M2 and M2_fert) were represented separately in the graph, indicating a strong

Table 2 Phylogenetic assignment of the *nif*H sequences of the tomato endophytic community

Cluster	Sequence count (%)[a]	OTU count (%)[b]	Distribution (%)[c]	Group	Closest cultivated taxa[d]
1J/1K	71.7	68.7	100	Alpha, Beta	Rhizobiales, Burkholderiales, Rhodospirillales
1A	11.2	8.3	75	Delta	Desulfuromonadales
1G	8.4	7	100	Gamma	Enterobacteriales, Pseudomonadales, Chromatiales, Methylococcales
1E	4	9.2	100	Firmicutes	Bacillales
3	3.1	3.3	100	Firmicutes, Delta	Clostridiales, Desulfovibrionales, Desulfobacterales
1B	1.2	2.2	100	Cyanobacteria	Oscillatoriales, Nostocales
1P	0.1	0.6	25	Beta	Rhodocyclales
1O	0.1	0.5	ND	Gamma	Chromatiales
1F	0.02	0.3	ND	Gamma	Pseudomonadales

[a] Percentage of sequences assigned to each *nif*H subcluster

[b] Percentage of operational taxonomic units (OTUs) classified in each *nif*H subcluster

[c] Abundance over the 6 samples analyzed (e.g., with the subcluster having a distribution of 100% among all samples)

[d] Orders closest to the predominant sequences observed in the subcluster; *ND*, not determined

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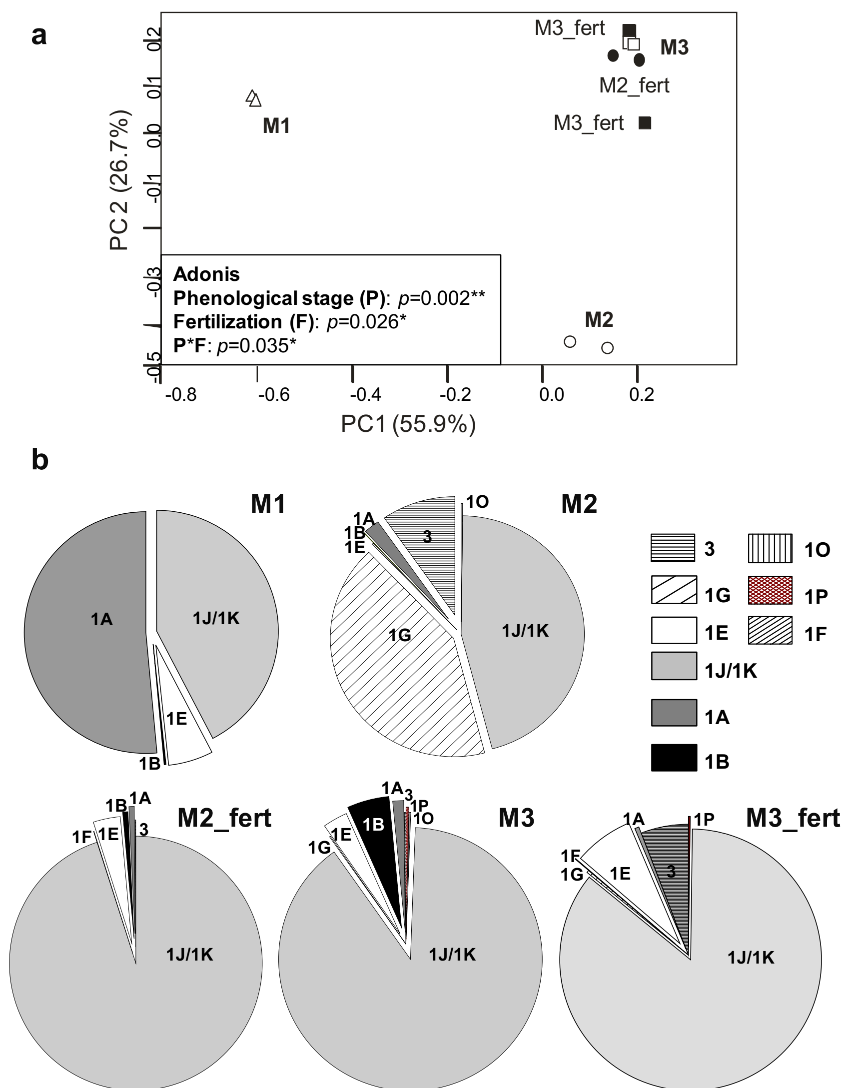

Fig. 3 Effect of fertilization and plant phenologic stage on EDC composition. Panel **a** Relationships among the EDCs were examined by principal coordinates analysis through the use of the Bray–Curtis dissimilarities. The phenologic stage is indicated by the symbols: M1, triangles; M2, circles; and M3, squares; with the white and black symbols denoting the absence and presence of fertilization, respectively. *p* values, Adonis (999 permutations), are shown for phenological stage (P), fertilization (F), and interaction (P*F). The first component (the *abscissa*) covers 55.9% and the second component (the *ordinate*) is the additional 26.7% of the variation. Panel **b** The pie charts illustrate the *nif*H subcluster proportion in the different samples according to the phenologic stage and fertilization conditions. A key to the textures indicating the various taxa is located in the upper right corner of the panel

compositional difference, while no such effect was observed in senescence (M3 and M3_fert).

There were clear differences in the proportion of *nif*H subclusters with respect to treatment or phenological stage (Fig. 3b). Plants in the vegetative stage (M1) were unique in their high proportion of subclusters 1A, but also 1J/1K and 1E, while samples of the nonfertilized flowering plants (M2) were mainly composed of sequences associated with subclusters 1G, 1J/1K, and 3. Fertilized samples (M2_fert, M3_fert) and nonfertilized senescent plants (M3) were characterized by a dominance of the subcluster 1J/1K (85% to 95% of the total sequences per sample). Of interest to us was that this analysis also revealed an association between subclusters having a low representation (less than 1%) and the developmental stages or fertilization conditions: for example, 1P (Rhodocyclales) was found in senescent plants, whereas 1F (Pseudomonadales) and 1O (Chromatiales) sequences were detected in only fertilized and nonfertilized plants, respectively (Fig. 3b).

About 84% of the total tomato *nif*H sequences were assigned to 13 of the most abundant OTUs (> 100 reads) and distributed in 6 *nif*H subclusters. The distribution of these phylotypes among the samples revealed key genotypes that maintained associations with the fertilization or the growth stage (Fig. 4). OTUs of subclusters 1J/1K, 1A, and 1E—related respectively to *Rhizobium phaseoli*, *Geobacter lovleyi*, and *Paenibacillus graminis*—were found in greater abundance in M1 samples than in late stages of growth. In contrast, the remaining 1J/1K OTUs related to uncultured Betaproteobacteria sequences and *Burkholderia* spp. were found in all samples except M1. In particular, OTU 204, representing 36.6% of the total database, underwent a marked increase in fertilized and senescent plants. Conversely, OTU 338, related to *Cyanothece* sp. (1B), was detected in only nonfertilized plants. In addition, associations between the fertilization and the plant phenologic stage were observed—e.g., the

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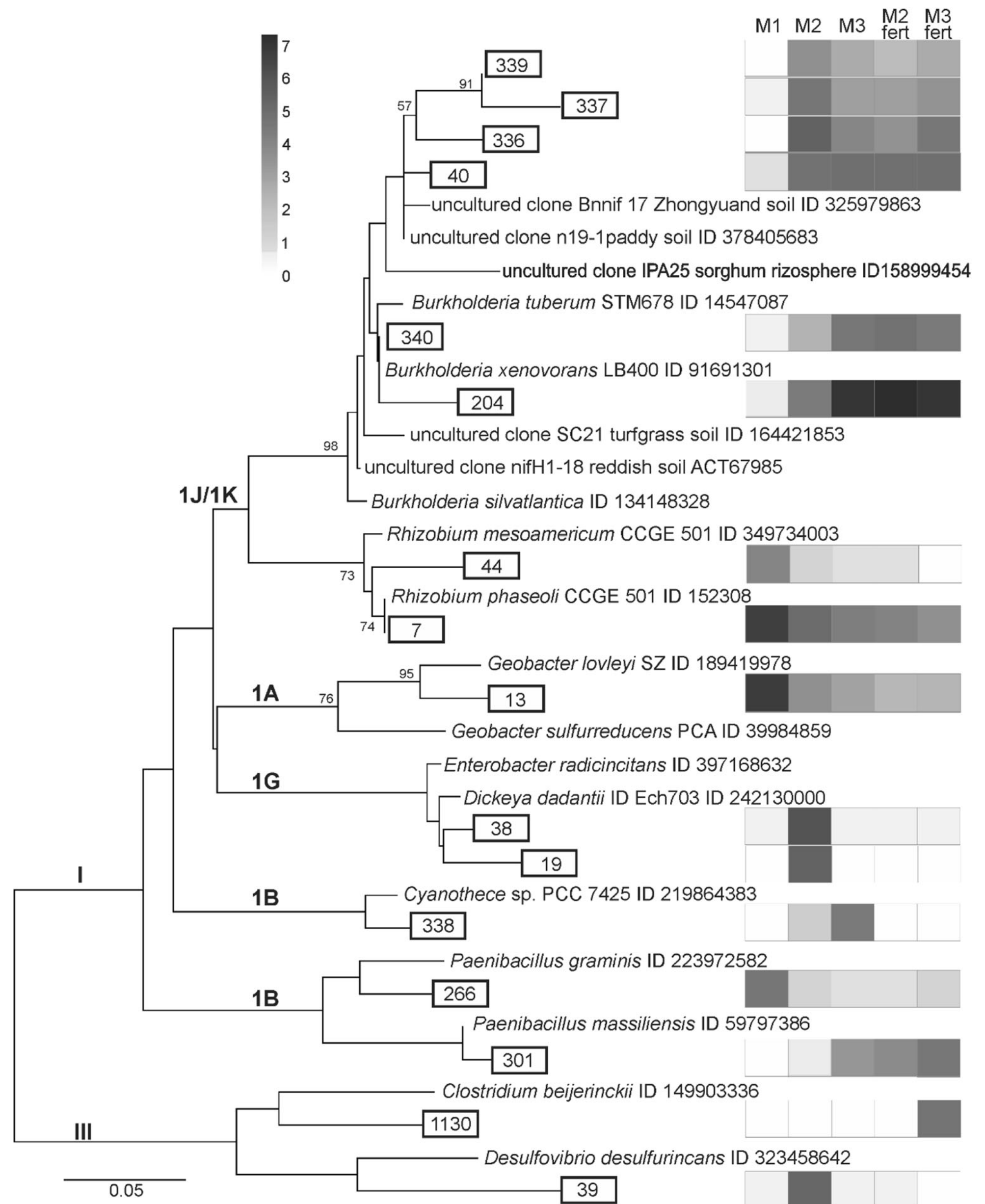

Fig. 4 Phylogenetic relationships among the most abundant *nif*H OTUs of the EDC based on neighbor-joining analysis of partial amino acid sequences. The numbers in the boxes identify the abundance of the OTUs observed in this study. The *nif*H subclusters are indicated to the left in the figure. Only bootstrap values > 50% are presented. The right side of the diagram illustrates the relative abundance of the sequences in the different samples according to the phenologic stage and fertilization conditions. The key to the textures in these horizontal strips is located above the uppermost one

OTUs included in the subgroups 1G and 3, related respectively to *Dickeya dadantii* and *Desulfovibrio desulfuricans*, constituted a high proportion of the total in nonfertilized flowering plants (M2), while the OTU related to *Clostridium beijerinckii* (subgroup 3) became associated with fertilized senescent plants (M3) (Fig. 4).

Discussion

In recent years, greenhouse tomato production has increased due to the high-yield potential and the significant consumer demand for year-round fresh produce [37]. High exogenous chemical fertilization, including nitrogen compounds, is commonly used in these systems [38, 39]. In the present work, we have examined the effect of phenologic stage and fertilization on the endophytic community in tomato through a metataxonomic approach while focusing our analysis on the endophyte populations with functional nitrogen-fixing capabilities.

Our findings revealed that the EDC of tomato is phylogenetically diverse with an abundance of different groups, such as Alpha-, Beta-, Gamma-, and Deltaproteobacteria plus Bacilli and Clostridia. Upon examination of other plant species, *nif*H phylotypes were observed in association with maize [40], rice [41, 42], sorghum [43], and perennial grasses [14] with the proteobacterial sequences being the most abundant in plant tissues as well as in the soil [29, 44] and the rhizosphere

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[cf. the review in 45]. At the taxonomic level, the number of *nif*H OTUs we obtained from the roots and stems of tomatoes was markedly higher than previously observed in tomato leaves using the 16S RNA as a gene signature [18, 19], suggesting that leaves represent a more restricted niche for endophytes and that the larger community in the rhizosphere should be amenable to establishing endophytic associations.

The structure of the EDC was affected by the phenologic state of the plant and by chemical fertilization, indicating a dynamic composition with populations that responded to environmental changes. Our results were in agreement with reports of endophytic bacterial communities in other plant species [13, 46]. By examining the rhizosphere of *Arabidopsis*, Chaparro et al. [47] reported changes in the relative abundance of major bacterial phylotypes throughout the different developmental stages of the plant. Similarly, working with the cultivable endophytic bacteria of wheat, Robinson et al. [48] observed differences in the EDC composition depending on the tissue type, the growth stage, and soil fertilization.

Of interest to us was that the composition of the EDC of plantlets was significantly different from that of the mature stage and furthermore varied upon growth in fertilized and nonfertilized soil particularly in the flowering stage. Indeed, group 1G increased in the flowering plants, while 1J/1K did so in that stage after fertilization (Fig. 3b). This succession of endophytic populations could be reflecting hormonal and/or physiologic changes or even the differing nutrient distribution that occurs with the age of the plant.

Chemical fertilization negatively affects *nif*H diversity as reflected by the profile of predominant phylotypes. For example, the predominance of *Burkholderia* spp. after transplantation, particularly in fertilized or senescent plants, could be related to the ecologic characteristic of the species of this genus that act as r-strategists—i.e., with high growth rates that confer the ability to compete and predominate in disturbed environments or with high levels of nutrients [49, 50]. By using similar primers and sequencing strategies, previous studies in our laboratory have indicated *Burkholderia* to be a normally non-abundant component of the potential and active soil diazotrophic community [28, 29]. The abundance of these phylotypes in the endophytic community of tomato, as well as in different tissues of several plants—such as rice, coffee, maize, and sugarcane [51–54]—prompts the speculation that this genus plays a significant role in N_2 fixation during its association with plants.

In contrast to *Burkholderia*, a reduced abundance of *Geobacter* and Cyanobacteria sequences was found in unfertilized plants. The relative abundance of *Geobacter* and *Anaeromyxobacter* has been shown to decrease in soils under long-term chemical fertilization, which has been related to its oligotrophic characteristic [50]. *Geobacter* species fulfill essential metabolic functions in anaerobic environments such as aquatic sediments, wetlands, rice paddies, and subsurface niches [55] as well as being active components of the N_2-fixing community in different soils [28, 56]. Nevertheless, the occurrence of *Geobacter* as endophytes is mostly restricted to flooded plant roots [17, 57]; although as a diazotroph, it was found as one of the most abundant inside leaves and stem of perennial grasses [14]. In line with this finding, our analysis also revealed sulfate-reducing bacteria, assigned to the genus *Desulfovibrio*, which appear to be common in waterlogged soils but rare in the endophyte community [17]. Conversely, N-fixing Cyanobacteria have been found to colonize different plant organs, intracellularly in the angiosperm *Gunnera* and extracellularly in species of liverwort, hornworts, Cycads, and Azolla [58]. Recent metagenomic analyses have shown a diversity of cyanobacterial phylotypes as common endophytes of other important agronomic species such as rice, wheatgrass, and Jingbai pear trees [57, 59–61]. Our results could be indicating that the multiplication of Cyanobacteria inside plants is favored under conditions of nutrient limitation. Similarly, we have previously found that cyanobacterial *nif*H abundance is high in soils with low levels of organic carbon, nitrogen, and phosphorus [28]. We would also emphasize that current knowledge on the plant colonization of Cyanobacteria and the subsequent maintenance of host interactions is based primarily on studies of similar types of symbiosis [62].

Geobacter and phylotypes related to Rhizobiales were present in abundance in tomato seedlings before transplantation, which finding indicates that these taxa could constitute seed-borne endophytic bacteria. Rhizobia have been reported to be a ubiquitous endophyte of several non-leguminous crops [18, 19, 45, 63]. In addition, rhizobial *nif*H gene expression was reported in association with rice and sugarcane [4, 57]. We were interested in the finding that sequences related to Rhizobiaceae and Bradyrhizobiaceae were predominant components in the different stages of plant growth and with and without fertilization. In particular, the detection of OTU 7 related to *Rhizobium phaseoli* occurred in all samples and was the most abundant OTU of the database (10.3% of the total sequences). Ubiquity and predominance, particularly in the first phenological stage, position this diazotroph as an interesting target for isolation and subsequent study of its potential as a tomato inoculant.

The phylogenetic diversity of endophytic bacteria revealed by our work may indicate the occurrence of different mechanisms of plant growth promotion in addition to biologic nitrogen fixation [1, 2]. The question is whether the high diversity reflects an N-poor environment inside the plant or, alternatively, whether this community performs beneficial functions other than biologic nitrogen fixation. Previous work has described the high proportion of diazotrophic endophytes in rice, which observation suggested an N-poor environment inside the plant [57]. Because the environment inside plant tissues is distinctive in terms of biotic and abiotic parameters that may influence the population dynamics of endophytic

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communities, our next challenge should be to characterize the methaphenome of such an environment—i.e., the link between bacterial functional expression (such as nitrogen fixation) and the resources within the plant environment [64].

Conclusion

The succession of nitrogen fixers throughout the different stages of growth includes taxa related to genera previously reported as plant endophytes—such as *Rhizobium*, *Burkholderia*, *Paenibacillus*, or *Anabaena*—but also novel and rare components that are mostly found in soil and anaerobic environments such as *Clostridium*, *Desulfovibrio*, or *Geobacter*. The abundance of rhizobia in all the phenologic stages of the tomato constitutes evidence that those bacteria are essential components of the EDC of non-leguminous plants, which observation led us to speculate that rhizobia exhibit growth-promoting activities other than nitrogen fixation with significant benefits to an agrosystem. We concluded that the plant growth stage is an essential parameter that promotes shifts in the composition of the EDC of tomatoes.

From an ecological context, species diversity, and function are important components of the sustainable agriculture systems. Our results provide evidence that exogenous chemical fertilization promotes adverse changes in the diversity and the profile of the otherwise predominant diazotrophic taxa. Populations such as *Burkholderia*, Cyanobacteria, and *Geobacter* would appear to be particularly affected by fertilization. These compositional changes could be a reflection of the nutritional status of the plant and/or the actual composition of the soil microbiome. Overall, considering together our findings on the endophytic population as well as previous data on soil bacteria [28, 50], they evidence the potential effects of excessive exogenous fertilization on the diversity of the N-fixing community.

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Code availability Not applicable.

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Compliance with ethical standards

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

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