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PLANT MICROBE INTERACTIONS

Alterations in the Endophyte-Enriched Root-Associated Microbiome of Rice Receiving Growth-Promoting Treatments of Urea Fertilizer and *Rhizobium* Biofertilizer

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Abstract

We examined the bacterial endophyte–enriched root-associated microbiome within rice (*Oryza sativa*) 55 days after growth in soil with and without urea fertilizer and/or biofertilization with a growth-promotive bacterial strain (*Rhizobium leguminosarum* bv. *trifolii* E11). After treatment to deplete rhizosphere/rhizoplane communities, washed roots were macerated and their endophyte-enriched communities were analyzed by 16S ribosomal DNA 454 amplicon pyrosequencing. This analysis clustered 99,990 valid sequence reads into 1105 operational taxonomic units (OTUs) with 97% sequence identity, 133 of which represented a consolidated *core* assemblage representing 12.04% of the fully detected OTU richness. Taxonomic affiliations indicated Proteobacteria as the most abundant phylum (especially α- and γ-Proteobacteria classes), followed by Firmicutes, Bacteroidetes, Verrucomicrobia, Actinobacteria, and several other phyla. Dominant genera included *Rheinheimera*, unclassified *Rhodospirillaceae*, *Pseudomonas*, *Asticcacaulis*, *Sphingomonas*, and *Rhizobium*. Several OTUs had close taxonomic affiliation to genera of diazotrophic rhizobacteria, including *Rhizobium*, unclassified *Rhizobiales*, *Azospirillum*, *Azoarcus*, unclassified *Rhizobiaceae*, *Bradyrhizobium*, *Azonexus*, *Mesorhizobium*, *Devosia*, *Azovibrio*, *Azospira*, *Azomonas*, and *Azotobacter*. The endophyte-enriched microbiome was restructured within roots receiving growth-promoting treatments. Compared to the untreated control, endophyte-enriched communities receiving urea and/or biofertilizer treatments were significantly reduced in OTU richness and relative read abundances. Several unique OTUs were enriched in each of the treatment communities. These alterations in structure of root-associated communities suggest dynamic interactions in the host plant microbiome, some of which may influence the well-documented positive synergistic impact of rhizobial biofertilizer inoculation plus low doses of urea-N fertilizer on growth promotion of rice, considered as one of the world's most important food crops.

The sequence data discussed in this publication have been disposed in NCBI's Sequence Read Archive (SRA) and are accessible through BioProject accession PRJNA526033.

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Introduction

The endosphere of the entire plant microbiome is an exclusive microhabitat that creates a distinct niche requiring numerous adaptations for successful colonization and harbors microbial communities that differ somewhat from the neighboring communities that colonize the surrounding rhizosphere soil or as epiphytes on the surface of the same plant [18, 19, 42, 59, 63, 67, 72]. Root endophytic microorganisms are considered to be more direct in affecting host plant attributes in situ than are the less-invasive microbial counterparts that only colonize rhizosphere soil surrounding the roots and those on their rhizoplane surfaces. Thus, endophytes play a more important role in development, productivity, and nutrient cycling of host plants [22, 66]. Some *competent* endophytic microorganisms are plant growth–promoting rhizobacteria (PGPR) that can stimulate vegetative growth and reproductive capacity and improve plant health through one or more functional attributes including increased seedling vigor; photosynthetic efficiency and capacity; fixation of atmospheric nitrogen; production of growth-regulating phytohormones; enhanced efficiency in nutrient uptake of mineral nitrogen, phosphorus, potassium, and several micronutrients; altered metabolism of plant cell walls; cell signaling; localized cell enlargement and division affecting their vegetative and reproductive architecture; enhanced nutrient exudation, systemic defense, and stress tolerance; and protective biocontrol of pathogens [4, 5, 10–12, 21, 22, 24, 33, 35, 37, 47, 69, 78, 80, 81]. These plant activities are major driving forces creating ecological niches occupied by endophytic members of the plant-associated microbial communities [26, 27, 60].

To increase crop growth and yield, modern agricultural practices apply different management regimes such as innovation of new plant cultivars, crop rotation, soil amendments with organic and/or inorganic fertilizers, highly efficient microbial biofertilizers, pesticides, and various biocontrol agents. It has been proposed that these agricultural practices may result in dysbiosis of the plant-associated microbiome and thereby potentially impact on crop production [38]. A concern in this research area is the possibility that microbial inoculation may disturb the natural plant-associated microflora and deplete native bacterial species that are beneficial and perhaps even essential for the development of the plant host in a competitive ecosystem [65]. The rationale for this question is that since endophytic bacteria rely on the nutritional supply contributed by the plant host, any restriction or alteration affecting its nutritional status and growth physiology may consequently affect the abundance, diversity, and benefits of the endophytic community that it harbors and nutritionally supports [24, 31]. This possibility also raises other important ecological questions, including how changes in the endophytic community structure within the plant host affect its ability to respond to environmental perturbations, and vice versa. For example, biocontrol agents applied to crops in open fields can sometimes adversely affect non-target, beneficial microbial inhabitants as well [34]. Similarly, adverse effects of inoculation with genetically modified microorganisms on protozoan bacteriovory, increased carbon turnover, displaced indigenous beneficial *Pseudomonas* populations, and long-term suppression of fungal populations have been documented [49].

Rice (*Oryza sativa* L.) naturally harbors a diverse endophytic microbial community within tissues of its interior plant compartments [3, 18, 19, 26, 27, 37, 59, 63, 72, 74, 80, 83]. Previous reports on the rice microbiome have shown that its endophyte community membership is influenced not only by instinctive factors like soil type, drought stress, plant genotype, and its current stage of development but also by organic farming vs. "ecofarming" practices that utilize pesticides and other organic amendments, and mineral N (ammonium sulfate) fertilizer applications [3, 18, 19, 32, 38, 59, 73, 83].

An issue of ecological importance that can ultimately impact on the advancement of successful, sustainable agricultural practices for production of plants like rice is to what extent does biofertilizer inoculation with effective PGPR alone, with application of urea-N fertilizer alone, or with the simultaneous combination of both treatments alter the natural bacteriome that develops in close association with its roots. To the best of our knowledge, this specific topic has not been previously investigated. Here, we describe studies focused on two interrelated objectives to fill that gap. The first objective was to utilize the culture-independent analysis of prokaryotic 16S ribosomal DNA (rDNA) gene sequences to compare the richness and distribution of relative abundance among the diverse taxa of bacteria representing the pooled *whole* endophyte–enriched bacteriome of all detected operational taxonomic units (OTUs) that colonize rice roots grown in soil with and without these treatments vs. the assemblage of the consolidated *core* endophyte–enriched community whose OTU richness is reproducibly found within the plant's roots regardless of which treatment they have received (i.e., no treatment–specific community taxa). The second objective was to examine how the community diversity of endophyte-enriched bacteria in the rice root microbiome may be restructured by application of a low dose of urea-N fertilizer, inoculation with an effective rhizobacterial biofertilizer strain, or the simultaneous combination of both treatments to optimally promote the development of the same rice variety grown in the same soil under otherwise identical environmental conditions. These

objectives were designed to provide insights into the biological complexity of beneficial plant-microbe interactions, rice microbiome diversity, and associations with (bio)fertilizer treatments for enhancement of crop productivity.

We previously isolated the *Rhizobium leguminosarum* bv. *trifolii* endophyte biofertilizer strain E11 used in this microbiome biodiversity study from within surface–sterilized field-grown roots of ratoon rice intermingled with berseem clover [80]. This strain has performed well as a biofertilizer that boosts rice vegetative growth, grain and straw yields, and the agronomic N fertilizer efficiency in extensive inoculation tests conducted over many years in the laboratory, greenhouse, small experimental field plots, and many large scaled-up farmers' fields in the Egypt Nile Delta [79–81]. Major facets of its growth-promoting ability are its stimulated production of growth-regulating auxin and gibberellin phytohormones, phosphate solubilization activity, and improved seedling vigor with an enhanced root architecture that makes the rice plant a better *miner* of macronutrient and micronutrient uptakes and utilizations when their availability is limiting [4, 5, 81]. Commonly, a major statistically significant growth benefit to rice productivity in many replicated field inoculation trials results from the synergistic treatments of biofertilizer inoculation with rhizobial strains (including the E11 PGPR) plus application of small doses of urea-N fertilizer [79–81].

Materials and Methods

Development of Endophyte-Enriched Communities in Rice Roots Grown in Soil

Rice (*Oryza sativa* cultivar Giza 178) was grown at the Sakha Agricultural Research Station, Kafr El-Sheikh, Egypt, in a clay loamy alluvial soil sample that originated from annual Nile flood sediments over thousands of years. The soil had a pH of 7.99, 1.71% organic matter, electrical conductivity of 0.37 dS/m, % sand/silt/clay contents of 42.4/42.4/15.1, available N and P contents of 57 ppm and 8 ppm, and soluble ion contents of 1.23 mEq/100 g (Ca^{2+}), 2.14 mEq/100 g (Mg^{2+}), 0.86 mEq/100 g (Na^{+}), 0.29 mEq/100 g (K^{+}), 0.24 mEq/100 g (HCO_3^{-}), and 0.48 mEq/100 g (Cl^{-}). The soil sample was obtained from an agricultural field located at 31° 06′ 30″ north × 30° 55′ 48″ east. It had been used for centuries under sustainable crop rotation with various cereals (predominantly rice and maize), legumes (predominantly berseem clover, *Trifolium alexandrinum*), and fiber crops (predominantly cotton). The Giza 178 cultivar is currently used in rice farming in that area of the Nile Delta. It is a Japonica type recommended for both fertile and saline soils, is blast- and drought-resistant, has a 135-day duration of growth from seed soaking until harvest, produces a short grain with filling of 19.9 g/1000 grains, a 70.9% milling index, and a paddy grain yield reaching 10.7 tons/ha in experimentally conducted fields (vis-à-vis, the 2018 Egypt national rice productivity average of 6.3 tons/ha).

Rice seedlings were germinated and grown for 23 days in a greenhouse tray nursery established with the above-described unsterilized soil, then transplanted to pots filled with the same soil and amended with common treatments of calcium superphosphate (15% P_2O_5) and potassium sulfate (48% K_2O), simulating 360 kg fertilizer/ha and 240 kg fertilizer/ha. Distinctive replicated characteristics of the potted soil comprised the above N-free fertilizer amendments, irrigation with sterile water when needed, and temperature and illumination in the greenhouse (16–18 °C during 10 h of night darkness, and a midday temperature of 33–35 °C with 14 h of daylight).

To examine specific treatment-based alterations in the rice-associated microbiomes, endophyte-enriched communities were developed within rice roots grown in the potted soil under four different experimental conditions. The first community, named S1 (control), was obtained from uninoculated roots receiving no N fertilizer treatment. The second *treatment community*, named S2 (+N), was obtained from uninoculated roots receiving urea-N fertilizer at a growth activation dose simulating 20 kg N ha^{-1} applied at 15 days after transplantation. The third treatment community, named S3 (+E11), was obtained from roots inoculated with 1 ml of a pure culture containing 1.34 × 10^8 CFU of *Rhizobium leguminosarum* bv. *trifolii* strain E11 applied to the rhizosphere of each rice seedling at 5 days after transplantation. The culture for this inoculum was grown in yeast extract mannitol broth [75] at 28 °C for 3 days. The fourth treatment community, named S4 (+N +E11), was obtained from roots receiving the same dose of urea-N fertilizer plus E11 inoculation. Each treatment was replicated with three plants in each of three separate pots. Digital images of the potted plants were acquired before harvest, spatially calibrated, and their number of leaves, cumulative shoot heights, and shoot-inscribed convex hulls were analyzed by CMEIAS Bioimage Informatics software [15]; https://lter.kbs.msu.edu/abstracts/555].

Extraction of DNA

The plants were uprooted at 55 days following transplantation. The roots were washed under running tap water to remove soil particles, rinsed with sterile distilled water, and blotted dry. The root samples were then cut into 1-in. segments, preserved in 4% paraformaldehyde in 10 mM phosphate buffer (pH 7.2), and stored at 4 °C until use. The shoots were oven-dried at 70 °C for 48 h followed by 3 h at 105 °C and then weighed.

The preserved root segments were shaken with 1–2-mm glass beads in buffer to dislodge and deplete their residual rhizoplane–associated microflora [2, 58], then rinsed with buffer, blotted dry, and weighed. Equal groups of these

endophyte-enriched root segments (200 mg each) were sampled from the three pooled replicates grown with each of the different treatments. Samples were frozen in liquid nitrogen, then ground using a mortar and pestle. The PowerSoil® DNA Isolation Kit (Mo Bio, USA) was used for isolation of DNA. Purity and quantity of genomic DNA was determined using the NanoDrop® ND-1000 UV-Vis spectrophotometer.

Sequencing of the 16S rDNA Gene Amplicon

The taxonomic composition and structure of the four root endophyte–enriched communities were examined using the culture-independent method of 454 pyrosequencing of their 16S rDNA gene (method of choice available at the time of sequence analysis). This high-throughput sequencing technique provided the in-depth analysis and detection of rare OTUs to enhance the identification of microbial community membership [9]. The primer pair 5′-AYTGGGYDTAAAGNG-3′ (forward) and 5′-CCGTCAATTCMTTTRAGT-3′ (reverse) [71] were used to target the hypervariable V4 region of the bacterial 16S rDNA (denoted by *Escherichia coli* nucleotide positions 563–924) with sample-specific barcodes to multiplex the sequencing reaction. Equal quantities (10 ng) of each DNA sample were used as a template in PCR with the following final concentrations of reagents: 400 nM 563F (forward) primer, 400 nM 924R (reverse) primer, 1× FastStart High Fidelity PCR buffer (Roche, Cat No. 03553400001), 0.5 U of Taq polymerase (Roche), and 200 mM dNTPs. Each reaction was set up in triplicate and amplified using the following cycle: 94 °C/3 min, followed by 30 cycles at 94 °C/45 s, 56 °C/45 s, 72 °C/1 min, and a final extension at 72 °C/7 min. Successful PCR amplification products from triplicate reactions were loaded and run on a 1.2% (*w*/*v*) agarose gel, then pooled and cleaned with a Qiagen PCR purification kit (Cat No. 28104). Concentrations of purified DNA samples were determined using Quant-iT (Invitrogen) according to the manufacturer's protocol and then adjusted to equimolar amounts. Sequencing was performed on the 454 FLX system at Utah State University (Logan, UT, USA) using the Lib-A kit according to the manufacturer's instructions (454 Life Sciences, Branford, CT, USA).

Processing of 454 Sequence Data

Sequencing data were processed using the RDPipeline of the Ribosomal Database Project (RDP) [14]. In brief, the initial process was used to extract targeted amplicon reads that match both 563F primer with edit distance ≤ 2 and 924R primer with edit distance ≤ 1, de-multiplex reads by sample barcodes and to remove low-quality reads (cutoffs used: read quality score ≥ 20, number of ambiguity bases = 0, 200 bp ≤ read length ≤ 400). Chimeras produced de novo during rDNA amplification were identified and removed by the UCHIME algorithm tool using USEARCH (ver. 8.1) [17]. Taxonomic placements of reads were determined using the RDP Classifier [76] (training set no. 16). Reads that could not be assigned to either the *Bacteria* or *Archaea* domains or were assigned to chloroplasts were removed from further analysis. The remaining reads were aligned using the Infernal alignment tool [50] to RDP's bacterial 16S Infernal model [13] and then clustered into OTUs at 97% sequence identity using the RDP clustering tool with complete-linkage clustering.

Diversity Analysis of 16S rDNA Sequences

The OTU richness and distribution of relative read abundances were analyzed to compare the endophyte-enriched community structures, including their ranked distribution and relativization, indices of alpha diversity/evenness/dominance, pairwise (dis)similarities and statistically significant differences in their beta diversity, multivariate cluster and ordination analyses, recognition of OTUs unique to each community, and assessment of OTUs assigned to bacterial taxa with potentially phytobeneficial characteristics. Ecological statistics were computed using PAST (ver. 3.21) [25], StatistiXL (ver. 1.10) [57], Species Diversity and Richness (ver. 4.1.2) [61], and Community Analysis Package (ver. 5.3.3.472) [62].

Results and Discussion

Plant Growth

Online Resource ESM1_figure1.pdf is a color image showing the typical growth response of potted rice plants receiving the different treatments. These above-ground differential growth responses expressed at this vegetative stage were supported by quantitative image analysis and dry weight data presented in Online Resource ESM2_table1.xlsx. Compared to the untreated control (S1), all three agronomic treatments significantly increased plant vegetative growth, with the S4 community receiving the combined treatment of E11 inoculation plus the urea-N fertilizer evoking the greatest positive growth response. These results indicated that the rice plants used to develop the endophyte-enriched microbiomes for this study were grown under a greenhouse simulator of conditions that permitted the expression of differential growth enhancement by application of the 4 tested experimental treatments, and this trend reflected the typical above-ground positive vegetative growth enhancement of rice initiated and developed by (bio)fertilization with the rhizobial E11 PGPR strain plus the tested doses of urea-N fertilizer under field

conditions prevailing during the early stage of rice growth in the Nile Delta [79, 81].

Data Recovery of 16S rDNA

The initial processing of sample de-multiplexing and primer matching yielded a total of 107,353 sequence reads. The quality filtering procedure reduced this number to 104,092 reads, representing a 3.0% reduction in size. Further processing using the chimera filtering tool reduced the number of reads by 2741 (2.6%) to an output of 101,351 reads (94.4% of the original 107,353 reads, 18,797 of which were unique). Inspection of the classification results to remove chloroplast reads and other reads that could not be confidently assigned to either *Bacteria* or *Archaea* domains plus alignment and clustering of the read sequences at the 0.03 distance level resulted in recognition of the final whole root endophyte–enriched microbiome that clustered into 1105 OTUs containing a total of 99,990 valid sequence reads. The average length of these final high-quality sequences ready for community analysis was 249 bases.

Online Resource ESM3_table2.xlsx is the master matrix of community membership data for the 1105 bacterial OTUs and their abundance of valid reads found in the rice root endophyte–enriched microbiome. Its rows indicate each OTU's assigned identification number and closest affiliated taxon (at genus-level resolution when possible) recognized with ≥97% sequence identity, their taxonomically ordered lineage (domain (bacteria)_phylum_class_order_family_genus), the number of valid reads in the column-defined whole microbiome, the consolidated core assemblage containing only those OTUs consistently found in all 4 individual communities (filled in orange), the untreated control community (S1), and each of the 3 individual treatment endophyte–enriched communities (S2, S3, S4). Online Resource ESM3_table2.xlsx also includes three groups of relative read abundances for each of the OTUs in the same untreated control and 3 treatment communities, including a *column-based* designation of the calculated % read abundances for each OTU relative to all reads in the same community, a *row-based* designation of the calculated % of read distributions for each OTU among the 4 individual communities, and a 2nd row-based designation of the normalized relativization of read abundances for each OTU within the 4 individual communities. All values reported in the column-based and 1st row-based indications of relative abundances sum to 100%. In the 2nd row-based analysis, a relativization adjustment to the maximum value is performed; i.e., the read values for the OTUs in 3 of the 4 individual communities in the same row are divided by the maximum number of reads for the same OTUs so their magnitude values are expressed relative to 1.0000.

Comparative Analysis of the Whole Microbiome vs. the Consolidated Core Assemblage

Online Resource ESM4_table3.xlsx further summarizes the total and proportional abundance of OTUs and their relative reads for the whole endophyte–enriched microbiome and its derived subset representing the consolidated core assemblage. The overall % proportional dissimilarity in OTU composition between the pairs of community assemblages was 8.27%. The Shapiro-Wilks normality test indicated that the read abundances among their OTUs were not normally distributed. Three corresponding non-parametric statistical tests (Kolmogorov-Smirnov, Anderson-Darling, Epps-Singleton) all rejected the null hypothesis of their equal distribution. The non-parametric Kruskal-Wallis statistical test indicated that the sample medians of their distributions were significantly different, with a $p < 0.000$ probability that those differences were due to chance.

Next, statistical modeling of OTU distributions in the whole microbiome and consolidated core community were examined by the Whittaker ranked abundance test (Online Resource ESM5_figure2.pdf). This analysis showed indistinguishable ordering of their ranked abundances for the first top 20 ranked OTUs (totaling 74,447 reads), a reduced number of reads for the next 113 ranked OTUs (totaling 17,211 reads), and 972 OTUs present in the whole microbiome that were found in only some of the 4 individual communities (hence not included in the core assemblage) as an early indication of a treatment-dependent microbiome alteration. Based on the chi-square goodness-of-fit tests, the data that produced both curves are best described by the mathematical model of a *truncated log-normal distribution*, which has characteristic truncated veil lines assigned to many rare OTUs [61]. The core assemblage is more truncated because it lacks numerous rare OTUs present in the whole microbiome (which inter alia, included 366 singletons). Ecological theory indicates that this best-fit truncated log-normal model is most applicable in situations where the natural community is very large, mature, very heterogeneous, and a consequence of many independent environmental factors acting multiplicatively on the distribution of species abundances and commonly fits well with most real-world diverse community data [29, 40, 44, 45]. Statistical tests of the same ranked abundance data rejected the *geometric series* model (niche preemption/resource apportionment model usually dominated by a few species in harsh species-poor environments or during early successional stages), the *log-series* model (occurring when only one or a few factors dominate the ecology of the community that represents a neutral biologic assemblage governed by stochastic immigration, emigration, birth, and death processes rather than by the occurrence of interspecific interactions like competition, predation, and other biotic interactions), and the uncommon *broken-stick* model (negative exponential distribution) that develops

when a maximum equitable distribution of nutrient resources is available and evenly apportioned among the community's constituent species [29, 44, 45].

It is noteworthy to emphasize that finding OTUs with relatively low abundances in these endophyte-enriched communities does not necessarily imply their unimportance nor indicate that they lack a significant functional contribution to community ecophysiology. Quite the contrary, rarity among class memberships in microbial communities can be *conditional* [64] since low relative abundance taxa can represent viable components that actively occupy unique ecological niches in the community and can make significant contributions to its stability and resilience, especially following environmental perturbation [20, 28, 64].

Several analyses were done to compare the levels of OTU heterogeneity in the whole microbiome vs. the consolidated core assemblage. The first of these was a Renyi diversity ordering test [56] to compare the relative magnitude of OTU heterogeneity across a range of entropy-based diversity indices [25, 61]. This analysis uses a family of indices rather than combining both species richness and their relative abundances into a single index [52, 61]. The Renyi analysis produced a plot of non-overlapping curves, indicating that the diversity ordering of those 2 community assemblages was robust and that the whole endophyte–enriched microbiome had a greater diversity than did the derived core community (Online Resource ESM6_figure3.pdf).

A more in-depth analysis was then performed to compare the levels of diversity for these two consolidated root endophyte–enriched assemblages. Table 1 summarizes the results, confirming that the diversity ordering was robust when examined by several diversity indices individually and that the value of each diversity index was greater for the whole microbiome than for the core community. The Solow permutation test [61, 68] and the bias-corrected percentile bootstrap random permutation test [25] were then performed to test if these differences in diversity indices were statistically significant. These two statistical tests equalize the sample sizes without data subtraction to the high, constant number of iterations (10,000 and 9999, respectively) when comparing the diversity indices of paired communities, thereby resolving the problem inherent to some diversity indices that are sensitive to unequal abundances of sampled individuals. The results indicated that the differences in diversity indices were statistically significant (Table 1).

The lower diversity in the consolidated core assemblage was further indicated by its significantly higher Berger-Parker dominance and Simpson dominance indices (Table 1) that embrace the proportional abundance of the most abundant species, and by the differences in position of their curves in a *K*-dominance analysis (Fig. 1) that depicts the percentage of total ranked OTU abundance against log OTU rank [61, 77]. The differences in height that separate the *K*-dominance curves for the core community and whole microbiome and the fewer OTUs needed to reach 100% of their total abundance (Fig. 1) indicate the greater dominance (hence, lower diversity) of the core community assemblage [39]. The taxon affiliation, dominance rank, and % cumulative contribution of reads for all 133 OTUs in the consolidated core community assemblage are listed in Online Resource ESM7_table4.xlsx.

The finding of the 133 OTUs in all four sampled endophyte-enriched communities independent/regardless of the agronomic treatments provides evidence of a replicated, common consolidated core component of shared OTU diversity in the rice endophyte–enriched microbiome and also provides supportive evidence of reproducibility in this 16S rDNA–based pyrosequencing community analysis.

Table 1 OTU diversity and dominance indices for the endophyte-enriched whole microbiome vs. consolidated core community associated with rice roots

Community diversity metric	Whole microbiome (W)	Consolidated core community (C)	Statistically significant result
Shannon-Wiener diversity	3.8350[a]	3.3690	W > C
Simpson diversity (1/*D*)	16.9700[a]	14.2800	W > C
McIntosh diversity	0.7596[a]	0.7378	W > C
Q statistic diversity	269.40[a]	28.94	W > C
Menhinick diversity	3.4940[a]	0.4393	W > C
Strong diversity	0.8412[a]	0.6594	W > C
Berger-Parker dominance	0.1502	0.1638[a]	C > W
Unbiased Simpson dominance	0.0589	0.0700[b]	C > W
Unbiased Shannon-Wiener diversity	3.835[b]	3.368	W > C

[a] Statistically significant greater value based on the Solow permutation test with 10,000 random partitions (at $p \leq 0.05$)

[b] Statistically significant greater value based on the bias-corrected percentile bootstrap random permutation test with 9999 iterations (at $p = 0.0001$)

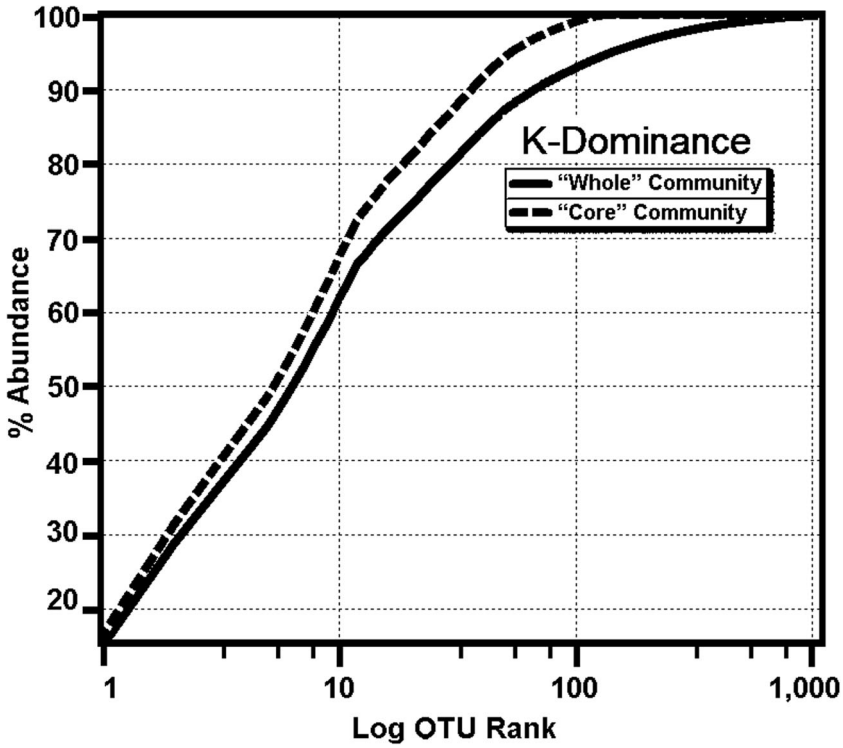

Fig. 1 *K*-dominance comparison of the rice root-associated endophyte-enriched whole microbiome (solid line) vs. consolidated core community (dash line)

Comparison of the Endophyte-Enriched Consolidated Core, Untreated Control and 3 (Bio)Fertilization Treatment Communities Associated with Rice Roots

Four methods were used to analyze the adequacy of community sampling effort for this portion of the endophyte-enriched root microbiome study. These included a rarefaction analysis (with 95% confidence envelope) of the consolidated core community, analysis of the progressive increase in Simpson 1/*D* and Shannon *H* diversity indices vs. cumulative number of OTUs found in the communities, and an OTU accumulation curve indicating the cumulative (not random average) number of OTUs scored individually from all four community assemblages plus the derived consolidated core community. Each analysis revealed characteristic ascending curves that eventually approached their maximal horizontal asymptote of saturation (Online Resource ESM8_figure4.pdf), suggesting that the pyrosequencing effort sampled these endophyte-enriched communities at a sufficient depth to capture most (if not all) of their OTU diversity. This equal sampling effort found an unequal number of OTUs for each community, some of which were present in all four individual communities (thus included in the consolidated core), and others were present in some but not all of the treatment communities, and still others were unique to each community (Table 2, Online Resource ESM3_table2.xlsx, Online Resource ESM9_table5.xlsx).

Table 2 summarizes the number of OTUs and their read abundances for the untreated control and the 3 individual treatment rice root communities in relation to the endophyte-enriched whole microbiome and consolidated core assemblages (their corresponding richness and abundance parameters of the individual OTUs are provided in Online Resource ESM3_table2.xlsx). Compared to the untreated S1 (control) community, these parameters were reduced in the treated communities within roots receiving the urea-N fertilizer (S2), the E11 biofertilizer (S3), and the combination of both treatments (S4). Application of urea-N fertilizer had a greater negative effect than the E11 biofertilizer, resulting in a reduction of these OTU-based community parameters. The number of OTUs and reads assigned to unique OTUs in the four individual endophyte–enriched communities (Table 2) is ranked in the following order: S1 (control) > S3 (+E11) > S4 (+N +E11) > S2 (+N). The taxonomic affiliation of these unique OTUs restricted to only one of the four individual communities is listed in Online Resource ESM9_table5.xlsx. These results clearly indicate alterations in the structure of the endophyte-enriched community assemblages residing within soil-grown rice roots that had received these agronomic treatments (urea-N fertilizer and/or E11 biofertilizer) to boost rice vegetative and reproductive productivity.

The OTU-based structures of the untreated control and 3 treatment communities were compared further. The Whittaker ranked abundance analysis (Online Resource ESM10_figure5.pdf) indicated patterns of their OTU distributions with similar descending slopes. The chi-square statistical test results indicated again that the *truncated log-normal distribution* model best fitted to these OTU distributions of ranked abundance data for each of the four communities. As

Table 2 Number of OTUs and their read abundances in the four individual endophyte–enriched communities associated with rice roots

Community parameter	S1 (control)	S2 (+N)	S3 (+E11)	S4 (+N +E11)
No. of OTUs (% of whole microbiome; 1105)	814 (73.67)	300 (27.15)	559 (50.68)	269 (24.34)
OTUs not in core community (% of whole microbiome)	291 (26.33)	805 (72.85)	546 (49.41)	836 (75.66)
OTU reads (% of whole microbiome; 99,990)	70,049 (70.06)	4113 (4.11)	22,277 (22.28)	3551 (3.55)
Singleton OTUs (% of total OTUs in the same community)	193 (23.71)	32 (10.67)	109 (19.50)	33 (12.68)
Unique OTUs (% in the same community)	383 (47.05)	39 (13.00)	177 (31.66)	46 (17.10)
Unique OTU reads (% in the same community)	1233 (1.76)	47 (1.14)	311 (1.40)	73 (2.06)
% whole microbiome OTUs found only in the indicated community	34.53	3.53	16.02	4.16

indicated earlier, this specific distribution model applies to very large, mature, heterogeneous communities responding to multiple independent environmental factors [29, 40, 44, 45].

The Renyi diversity ordering analysis of the four community assemblages of OTUs produced robust, non-overlapping curves that ranked their diversity as S2 > S4 > S1 > S3 across the range of several entropy-based indices (Fig. 2). The right-tailed sum is another diversity ordering analysis that plots the proportion of the total community in descending order of each OTU's abundance. Communities containing many OTUs with similar abundance (high evenness) produce curves that descend slowly as the increasing number of OTUs is excluded from the summation (termed the *i* value), whereas communities heavily dominated by large abundances of just a few OTUs (high dominance) produce curves that decline more steeply as *i* increases [52, 61]. This right-tailed sum analysis assembled the four communities into two distinct groups (Online Resource ESM11_figure6.pdf). One group contained the two communities developed within roots that received the urea-N fertilizer [S2 (+N) and S4 (+N +E11)]. Those two communities had OTUs with similar abundance, hence higher evenness and diversity (Tables 2 and 3). The second group contained the other two communities within roots that did not receive the urea-N fertilizer [S1 (control) and S3 (+E11)]. Their curves declined more steeply due to the greater dominance (hence lower evenness and diversity) of their OTU distributions. The opposite *K*-dominance trend was lower for the OTU distributions in the two endophyte-enriched communities within roots receiving the urea-N fertilizer (Online Resource ESM12_figure7.pdf). This pattern resembled the S2 > S4 > S1 > S3 ranking obtained with several indices of diversity and evenness, plus the inverse ranking of S3 > S1 > S4 > S2 for dominance indices computed separately. All of these rankings reflect differences in OTU richness, distribution of abundance, evenness, and dominance that were statistically significant using the Solow permutation test with 10,000 iterations and the bias-corrected percentile bootstrap random permutation test with 9999 partitions (Table 3).

Considered collectively, these differences between the four individual endophyte–enriched communities provide evidence that help explain the statistically significant ranking of

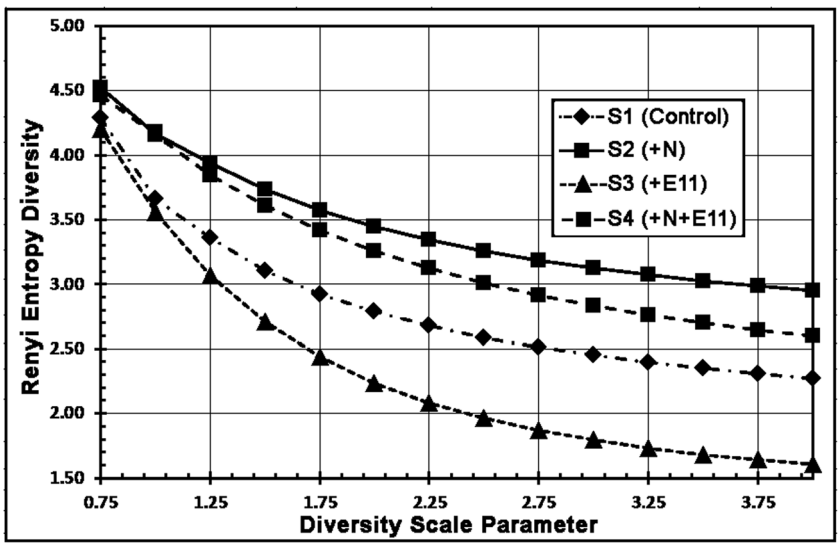

Fig. 2 Renyi entropy multiple community analysis to compare the robustness of diversity ordering between the 4 treatment endophyte–enriched communities associated with rice roots [S1 (control) diamond dot dash; S2 (+N) square solid line; S3 (+E11) triangle dash line; S4 (+N +E11) square dash line]

Table 3 Indices of diversity, evenness, and dominance computed from OTU abundance data for the untreated control endophyte–enriched community and the 3 treatment endophyte–enriched communities associated with rice roots receiving urea-N fertilizer and/or E11 biofertilizer

Community diversity metric	S1 (control)	S2 (+N)	S3 (+E11)	S4 (+N +E11)	Statistically significant ranking
Shannon-Wiener diversity	3.709^{a}	4.181^{b}	3.533^{c}	4.109^{d}	S2 > S4 > S1 > S3
Simpson diversity (1/*D*)	15.91^{a}	31.06^{b}	9.008^{c}	25.76^{d}	S2 > S4 > S1 > S3
McIntosh diversity	0.752^{a}	0.870^{b}	0.696^{c}	0.854^{d}	S2 > S4 > S1 > S3
Simpson evenness	0.0195^{a}	0.1035^{b}	0.0161^{c}	0.0958^{d}	S2 > S4 > S1 > S3
McIntosh evenness	0.7764^{a}	0.8702^{b}	0.6962^{c}	0.8544^{d}	S2 > S4 > S1 > S3
Smith and Wilson evenness	0.4128^{a}	0.6011^{b}	0.3474^{c}	0.5795^{d}	S2 > S4 > S1 > S3
Berger-Parker dominance	0.176^{a}	0.091^{b}	0.306^{c}	0.138^{d}	S3 > S1 > S4 > S2
Bias-corrected Simpson dominance (*D*)	0.06158^{a}	0.03161^{b}	0.10690^{c}	0.03813^{d}	S3 > S1 > S4 > S2
Bias-corrected Shannon-Wiener diversity	3.726^{a}	4.160^{b}	3.550^{c}	4.089^{d}	S2 > S4 > S1 > S3

Differences between values in the same row followed by a different letter (a, b, c, d) are statistically significant ($p \leq 0.05$) using the Solow permutation test with 10,000 random partitions. Row values for the bias-corrected Simpson dominance and Shannon-Wiener diversity indices (bottom 2 rows) were computed using the PAST percentile bootstrap random permutation test with 9999 iterations, and their differences are all statistically significant (a, b, c, d) (p ranges from 9.88×10^{-197} to 2.06×10^{-4} for the bias-corrected Simpson dominance index and p ranges from 1.81×10^{-111} to 4.94×10^{-2} for the bias-corrected Shannon Wiener diversity index)

their diversity with high scientific consistency and rigor (Fig. 2, Online Resource ESM8_figure4.pdf, Online Resource ESM11_figure6.pdf, Online Resource ESM12_figure7.pdf).

Next, three pairwise analyses were performed to assess the degree of (dis)similarity in taxonomic compositions of the communities hosted by rice roots responding to the different agronomic treatments. The first analysis ranked the number and relative (%) proportion of OTUs in some but not all individual specified communities (hence excluded from the consolidated core assemblage) as follows: S1 (control) (681; 83.66%) > S3 (+E11) (426; 76.21%) > S2 (+N) (167; 55.67%) > S4 (+N +E11) (269; 50.56%). The 2nd discriminating comparison involved a binary co-occurrence similarity analysis using the Jaccard, Ochigi, and Kulczynski coefficients (Online Resource ESM13_table6.xlsx). The latter two coefficients are useful in this analysis because they exclude binary double zeros in the paired similarity comparison. That exclusion avoids assigning a high level of similarity to communities that lack both species, which can become a critical problem in habitats that harbor a very large richness of species [40, 62]. The results of that 2nd analysis identified the pair of communities receiving the urea-N fertilizer [S2 (+N) ↔ S4 (+N +E11)] with the greatest overall similarity, and the pair of S1 (control) ↔ S4 (+N +E11) (no treatment vs. double agronomic treatment) with the least similarity.

The 3rd pairwise analysis revealed significant dissimilarities in the OTU-based structures of the endophyte-enriched communities within the rice roots responding to the various agronomic treatments (Table 4). Like the indices of diversity/evenness/dominance, each dissimilarity (distance) index summarizes a slightly different aspect of the community structure. Thus, the use of multiple distance indices can reveal how robust (high scientific consistency) is their ranking of dissimilarities in community structures. Also, each distance index varies in overall effectiveness when the abundances of sampled individuals are unavoidably dissimilar, with some being more affected than others [62]. For instance, the average Euclidian, Geodesic, and Canberra distance metrics are well adapted to abundance data and less influenced by different sample sizes compared to the Euclidian and Bray-Curtis distance metrics [40, 62]. The pairwise distance analysis indicated that the community pair with the *greatest exclusive dissimilarity* in structure was S1 (control) ↔ S4 (+N +E11), and the pair with the *least dissimilarity* was S2 (+N) ↔ S4 (+N +E11). These results concur with the other analyses indicating the greater similarity in structure between the two communities (S2 and S4) associated with roots receiving the urea-N fertilizer treatments, and also when compared to the untreated control, those treatments exert a greater dysbiosis of the root endophyte–enriched microbiome than does the E11 biofertilizer treatment.

We used three ordination methods of multivariate statistical analysis to further evaluate the (dis)similarity in OTU membership and their read abundances among the four individual endophyte–enriched communities compared to the consolidated core assemblage. This examination included an autoclassification using hierarchical agglomerative cluster analysis (Ward's increase in sum of squares using the average Euclidian distance metric), a multi-dimensional scaling analysis with 200 iterations, and a 2-dimensional principal covariance analysis. These methods reduce the dimensionality of the quantitative dataset by indicating the distance/proximity between points that reveal the (dis)similarity in OTU composition in the sampled communities. The results show that the

Table 4 Co-occurrence dissimilarity (distance) analysis of OTUs in each pair of endophyte-enriched communities associated with rice roots

Dissimilarity (distance) metric	S1 vs. S2	S1 vs. S3	S1 vs. S4	S2 vs. S3	S2 vs. S4	S3 vs. S4
Euclidian distance	16,978.4	13,978.3	17,008.6	7055.0	572.7	6825.8
Average Euclidian distance	510.76	420.51	511.67	212.23	17.23	205.34
Bray-Curtis distance	0.8952	0.5510	0.9104	0.7370	0.3770	0.7578
Geodesic distance	1.1920	1.4125	1.1915	1.5346	1.3617	1.0701
Manhattan distance	66,394	51,248	67,002	19,402	2906	19,522
Canberra distance	794.94	810.58	817.97	562.34	309.56	549.40

points assigned to the two communities in roots treated with urea-N fertilizer [S2 (+N) and S4 (+N +E11)] positioned closely to one another, indicating (again) their high similarity to each other and their greater dissimilarity to the other communities (Figs. 3 and 4, Online Resource ESM14_figure8.pdf).

The variable vectors plotted within the same reduced space as the sample points in the principal covariance analysis (arrows in Online Resource ESM14_figure8.pdf) identified OTU-10 (*Rheinheimera*) and OTU-17 (an unclassified *Rhodospirillaceae*) as the dominant OTU members that distinguish the structured ordinations of these endophyte-enriched communities. Together, these two abundant OTUs contributed 30.95% of the total reads in the consolidated core assemblage (Online Resource ESM3_table2.xlsx, Online Resource ESM7_table4.xlsx). Interestingly, although most described species of *Rheinheimera* are typically biofilm-producing aquatic bacteria, *Rheinheimera tangshanensis* was first isolated from washed roots of rice grown in soil [82] and also was subsequently found to colonize the rhizosphere of barley (*Hordeum secalinum*) [70]. Also potentially important here are the reports that some *Rheinheimera* species produce secondary metabolites and proteins with antimicrobial activities against a broad spectrum of microorganisms and produce bioactive metabolites that modify root elongation and morphology of host plants, mimicking indoleacetic acid action [8, 53].

Comparison of OTUs in the 4 Endophyte-Enriched Communities at 3 Levels of Taxonomic Resolution

Several approaches were used to examine the heterogeneity of bacterial taxa at the phylum, class, and genus levels of taxonomic resolution among the OTU members of the four individual rice root endophyte–enriched communities. The seriation presence-absence matrix [7] provides a quick visual inspection of the phylum affiliations represented by bacterial members shared in all four communities, plus the phyla that are uniquely represented in some but not all four communities. This evaluation was followed by a ranking of phyla based on the distribution of their OTUs and by a vertical stacked bar plot derived from those data indicating the cumulative and relative % abundances of OTUs among the represented phyla for each of the four individual communities and the consolidated core community assemblage.

Eighteen phyla were identified in the taxonomic lineages among the bacterial OTU members found in the four individual communities (Table 5, Online Resource ESM3_table2.xlsx, Online Resource ESM15_figure9.pdf, Online Resource ESM16_figure10.pdf). Their ranked distributions of relative abundance were as follows: S1 (control) > S3 (+E11) > S4 (+N +E11) > S2 (+N). OTUs in the phylum Proteobacteria were most abundant in each of these communities, and that phylum also dominated the taxonomic lineage of bacterial OTUs in the consolidated core assemblage

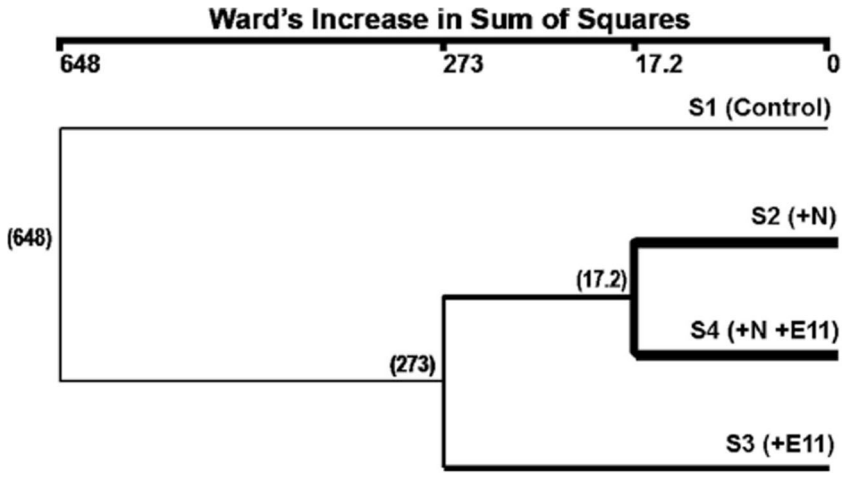

Fig. 3 Dendrogram of (dis)similarity relationships among the OTU structures of the consolidated core assemblage and the 4 individual endophyte–enriched communities developed in association with rice roots, with the line thickness weighted by the similarity within groups and the critical values (in parenthesis) at the fusion points reporting the distance values that caused the samples to be combined

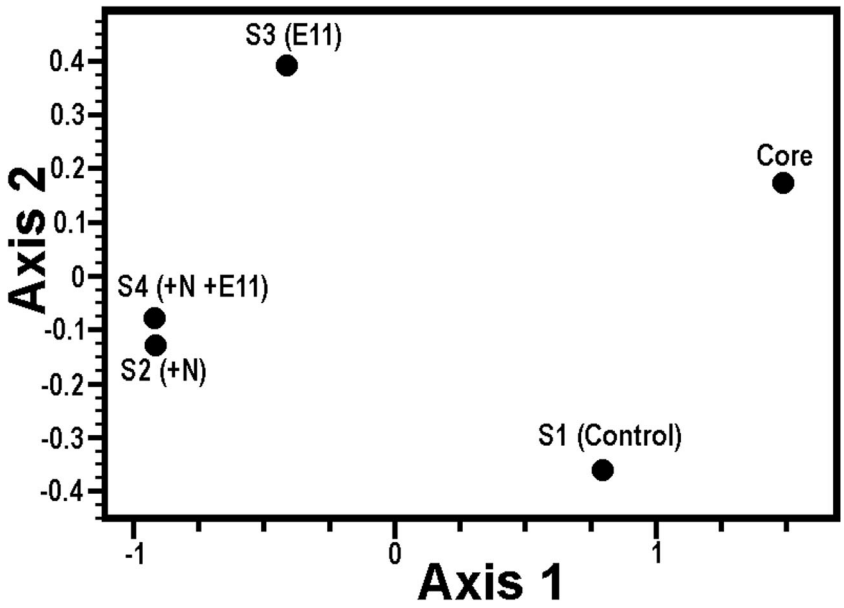

Fig. 4 Scatterplot of the 200-iterated multi-dimensional scaling analysis of (dis)similarities among the consolidated core assemblage and the 4 individual endophyte–enriched communities associated with rice roots

(Table 5, Online Resource ESM16_figure10.pdf). The ranked distributions of abundance among *class-level* lineage affiliations of OTUs in the phylum Proteobacteria for the endophyte-enriched whole microbiome, consolidated core community, and across the four individual communities were as follows: α-Proteobacteria > γ-Proteobacteria > β-Proteobacteria > δ-Proteobacteria > Oligoflexia (Table 6). Less than 1% of the Proteobacteria OTUs were unclassified. Other major represented phyla included Firmicutes, Bacteroidetes, Verrucomicrobia, and Actinobacteria. These bacterial phyla have been found previously in microbial communities colonized within the interior of various plants [3, 6, 18, 21, 22, 26, 27, 32, 35–37, 41, 46, 59, 63, 72–74, 81, 83]. Phyla of rare OTUs found in only one of the four individual communities included WPS-1 and Latescibacteria in S1 (control), BRC1 and Amatimonadetes in S3 (+E11), and Parcubacteria in S4 (+N +E11).

A total of 98.47% of the valid reads for the 1105 bacterial OTUs found in the whole endophyte–enriched microbiome clustered into 199 identified genera (Online Resource

Table 5 Distribution of OTU abundances (and their reads) for each bacterial phylum in the rice endophyte–enriched communities

Phylum	S1 (control)	S2 (+N)	S3 (+E11)	S4 (+N +E11)	Core assemblage
Proteobacteria	452 (64,078)	177 (3469)	295 (19,141)	156 (2897)	97 (84,266)
Bacteroidetes	58 (660)	22 (106)	31 (306)	23 (311)	8 (885)
Firmicutes	58 (2182)	26 (179)	38 (1378)	10 (91)	5 (3267)
Actinobacteria	40 (625)	16 (128)	45 (676)	12 (57)	9 (1151)
Chloroflexi	39 (114)	19 (25)	32 (59)	16 (34)	2 (31)
Verrucomicrobia	25 (714)	7 (95)	15 (180)	12 (68)	5 (538)
Acidobacteria	18 (100)	3 (5)	15 (50)	3 (10)	1 (31)
Gemmatimonadetes	11 (103)	5 (14)	5 (32)	4 (7)	2 (102)
Planctomycetes	5 (11)	0 (0)	4 (4)	3 (3)	0 (0)
Deinococcus-Thermus	5 (384)	2 (4)	7 (57)	2 (6)	1 (401)
Ignavibacteria	4 (12)	1 (1)	2 (6)	1 (1)	0 (0)
Cyanobacteria	1 (1)	0 (0)	2 (2)	1 (1)	0 (0)
Spirochaetes	1 (62)	0 (0)	1 (1)	0 (0)	0 (0)
BRC1	0 (0)	0 (0)	2 (4)	0 (0)	0 (0)
WPS-1	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)
Latescibacteria	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)
Parcubacteria	0 (0)	0 (0)	1 (2)	0 (0)	0 (0)
Armatimonadetes	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)
Unclassified bacteria	95 (1000)	2 (87)	63 (378)	26 (65)	3 (986)

Table 6 Distribution of class-level subdivisions among Proteobacteria OTUs in the whole microbiome, consolidated core assemblage, and 4 individual endophyte–enriched communities associated with rice roots

Proteobacteria subclass	Whole microbiome	Consolidated core assemblage	S1 (control)	S2 (+N)	S3 (+E11)	S4 (+N +E11)
All Proteobacteria	555 (89,585; 100)	97 (84,266; 100)	452 (64,078; 100)	177 (13,469; 100)	295 (19,141; 100)	156 (2897; 100)
Alphaproteobacteria	309 (48,748; 54.4)	52 (46,227; 54.9)	266 (39,042; 60.9)	90 (1728; 49.8)	161 (16,722; 35.1)	82 (1256; 43.4)
Betaproteobacteria	53 (2654; 3.0)	16 (2175; 2.6)	39 (1702; 2.7)	27 (139; 4.0)	37 (645; 3.4)	24 (168; 5.8)
Gammaproteobacteria	134 (37,902; 42.3)	28 (35,830; 42.5)	106 (23,145; 36.1)	52 (1588; 45.8)	76 (11,727; 61.3)	43 (1442; 49.8)
Deltaproteobacteria	32 (159; 0.2)	0 (0; 0)	22 (125; 0.2)	4 (16; 0.2)	13 (25; 0.1)	3 (13; 0.1)
Oligoflexia	1 (9; 0.01)	0 (0; 0)	0 (0; 0)	1 (1; 0.03)	1 (2; 0.01)	1 (6; 0.2)
Unclassified Proteobacteria	26 (113; 0.1)	1 (34; 0.1)	19 (64; 0.1)	3 (7; 0.2)	7 (20; 0.1)	3 (22; 0.8)

Values indicate the number of Proteobacteria OTUs (their read abundances; % of all Proteobacteria) in each community

ESM3_table2.xlsx). The taxonomic affiliations of genera among the 133 OTUs in the consolidated core assemblage and the 615 unique OTUs in the four individual communities are indicated in Online Resource ESM7_table4.xlsx and Online Resource ESM9_table5.xlsx, respectively. Online Resource ESM17_figure11.pdf indicates the % cumulative distributions of ranked read abundances for the 10 most plentiful OTUs in the untreated S1 control community (collectively equal to 55.48% of its 70,049 total OTU read abundances) compared to the same OTUs in the 3 treated communities. The extents to which the 6 paired distributions (S1 vs. S2, S1 vs. S3, S1 vs. S4, S2 vs. S3, S2 vs. S4, S3 vs. S4) of relative read abundances differed among these top 10 OTUs in those four communities were analyzed by the Kolmogorov-Smirnov, Anderson-Darling, and Epps-Singleton non-parametric statistical tests (Online Resource ESM18_table7.xlsx). Their differences in distribution were statistically highly significant between all community pairs except for the couple that received the urea-N fertilizer treatment [S2 (+N) and S4 (+N +E11)], consistent with the overall closer similarity of these latter two communities as indicated earlier. These (dis)similarities in distribution of abundance among the 10 top genera in all four communities are also indicated in the cluster analysis dendrogram presented in Online Resource ESM19_figure12.pdf.

This study also explored the heterogeneity in distribution of endophyte-enriched OTUs among the genera known to contain diazotrophic (N_2-fixing) species that associate with plant roots, including rice [1, 6, 12, 18, 22, 23, 35, 36, 43, 46, 48, 54, 55, 63, 69, 72, 74, 80, 81]. Overall, 14 genera

Table 7 Diversity and abundances among OTU reads with taxonomic affiliation to genera containing root-associated diazotrophs in the 4 individual endophyte–enriched communities

Taxon affiliation	No. of OTUs (no. of reads) per community				Total no. of OTUs (reads; % abundance)
	S1 (control)	S2 (+N)	S3 (+E11)	S4 (+N +E11)	
Rhizobium	12 (6844)	6 (213)	8 (1781)	5 (191)	12 (9029; 88.2)
Unclassified *Rhizobiales*	22 (321)	5 (7)	10 (60)	6 (7)	25 (395; 3.86)
Azospirillum	8 (255)	5 (20)	6 (81)	2 (16)	8 (342; 3.34)
Azoarcus	5 (107)	4 (13)	5 (25)	4 (13)	5 (158; 1.54)
Unclassified *Rhizobiaceae*	6 (108)	1 (3)	2 (38)	1 (1)	6 (150; 1.47)
Bradyrhizobium	1 (47)	0	1 (18)	0	1 (65; 0.64)
Azonexus	1 (37)	2 (2)	0	2 (3)	2 (42; 0.41)
Mesorhizobium	1 (11)	0	1 (3)	0	1 (14; 0.14)
Devosia	1 (10)	0	2 (3)	0	2 (13; 0.13)
Azovibrio	1 (11)	1 (1)	0	0	1 (12; 0.12)
Azospira	0	1 (1)	1 (6)	1 (1)	1 (8; 0.08)
Azomonas	2 (2)	0	0	0	2 (2; 0.02)
Azotobacter	1 (1)	0	0	0	1 (1; 0.01)

containing 71 OTUs with this characteristic were found here within the rice root microbiome. Table 7 summarizes their closest taxon affiliations, distributions in number, and relative % abundances of OTUs within each of the four individual communities. Their relative read abundances ranked as follows: *Rhizobium* > unclassified *Rhizobiales* > *Azospirillum* > *Azoarcus* > unclassified *Rhizobiaceae* > *Bradyrhizobium* > *Azonexus* > *Mesorhizobium* > *Devosia* > *Azovibrio* > *Azospira* > *Azomonas* > *Azotobacter*. OTUs of *Rhizobium*, unclassified *Rhizobiales*, unclassified *Rhizobiaceae*, *Azospirillum*, and *Azoarcus* were found in all four individual communities and therefore were included in the consolidated core assemblage. Sixty-two of the 71 OTUs of diazotrophic genera were found in the untreated S1 (control) community, and fewer proportions of OTUs were found in the three treatment communities [ranked as follows: S1 (control) > S3 (+E11) > S2 (+N) > S4 (+N +E11)]. The finding of major abundances among the rhizobial OTUs in the untreated S1 control community provides an additional solid line of experimental evidence indicating their natural endophytic association within roots of the rice plant, as previously reported [80, 81]. Also, this ranking of fewer diazotrophic genera of OTUs associated with rice roots receiving urea relates to previous studies indicating decreases in the diversity, population size, and nitrogenase activity of diazotrophic bacteria associated with rice roots receiving N fertilizer [51, 73].

Potentially important and consistent with previous findings [16, 30] is the detection of OTUs with taxonomic affiliation to genera containing species of potential human pathogens (e.g., *Vibrio*, *Legionella*, *Yersinia*, *Enterobacter*, *Clostridium*) within the rice root–associated endophyte-enriched microbiome, as indicated in Online Resource ESM3_table2.xlsx.

Finally, 149 OTUs (13.48%) with a cumulative abundance of 1530 reads (1.53%) found in the endophyte-enriched rice root microbiome had no match at the 97% sequence identity level to 16S rDNA sequences in the existing RDP database of described bacteria. The read abundances for these OTUs of unclassified bacteria are listed in Online Resource ESM3_table2.xlsx and Online Resource ESM20_table8.xlsx. Only 3 of these unclassified OTUs were found in all four individual communities, hence included in the consolidated core assemblage. These results indicate that the rice roots harbor genera of many different bacterial species that remain to be discovered, isolated, and described, and their impact on rice productivity should be considered in future studies.

Summary and Closing Statements

This study used bacterial 16S rDNA amplicon sequencing to define the natural endophyte–enriched root-associated microbiome of rice grown in an arable soil from the Nile Delta of Egypt, and how that community assemblage is restructured upon application of urea-N fertilizer and/or inoculation with an effective plant growth–promoting endophytic strain of *Rhizobium leguminosarum* bv. *trifolii*. The study revealed that soil-grown rice roots harbor a diverse endophyte–enriched bacterial core community and identified numerous cases in which that rice microbiome was altered in roots receiving these major agronomic treatments used for intensive rice production, suggesting that they impose selective forces that reshape the community assemblages within rice roots. 99,990 valid sequence reads were clustered at the 97% sequence identity level into 1105 different OTUs, with taxonomic affiliation to Proteobacteria as the most abundant phylum (especially α-Proteobacteria and γ-Proteobacteria classes) and into 199 genera dominated in read abundance by *Rheinheimera*, unclassified *Rhodospirillaceae*, *Pseudomonas*, *Asticcacaulis*, *Sphingomonas*, and *Rhizobium*. The richness and relative read abundances of OTUs were significantly reduced in endophyte-enriched communities receiving urea-N fertilizer and/or biofertilizer treatments compared to the community associated with control roots receiving neither of these treatments. Also, several unique OTUs were enriched in each of these individual communities. The information gained adds a significant new dimension to our understanding of this important natural plant-microbe interaction and sheds light on the range of impacts associated with the agronomic treatments of *Rhizobium* biofertilizer inoculation and urea-N fertilizer applications on those rice community structures. The biological complexity and staggering diversity of these remodeled communities profoundly influence how we should contemplate the underlying biological interactions in beneficial plant-bacteria associations that result in plant growth promotion. These findings raise the provocative question of whether all the benefit of urea application should be attributed to satisfying the nutritional demand of the crop plant for N, or could its restructuring of the root endophyte–enriched community assemblage as revealed here contribute to creating the optimal blend of community members that evoke the positive growth responses? This study also elevates significant questions about the real-world biological variables that must be considered during efforts to manage plant stresses imposed by climate change, infertile/alkaline/saline soils, and pest/pathogen outbreaks, plus to elucidate the mechanisms (resulting from 2414+ differentially expressed genes [78]) that biofertilizers of rhizobial PGPR graciously deliver to improve the productivity of rice, considered as one of the world's most important cereal crops.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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