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ORIGINAL ARTICLE

Abundance and diversity of endophytic and rhizospheric diazotrophs associated with rice roots from different rice rotation systems under field conditions

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Abstract
Biological nitrogen fixation contributes greatly to the sustainability of agroecosystems. However, more information is needed about the impact that agricultural intensification, a frequent practice nowadays, would have on diazotrophic communities associated with plants. This work addresses the influence of intensifying rice cropping systems on the abundance, diversity, and structure of diazotrophic communities associated with rice roots (rhizospheric and endophytic bacteria) from a field experiment. Three different rice rotation systems (rice–pasture, rice–soybean, and continuous rice) at two crop growth stages (TBF: tillering before flooding and FF: flowering-flooded) were studied. The results showed that the rhizospheric soils had the greater *nifH* gene abundance, and the abundance and diversity of rhizospheric and endophytic diazotrophic communities significantly increased at the FF stage. Conversely, *nifH* abundance in bulk soils remained unaltered. Phylogenetically and metabolically diverse diazotrophic communities were found to be associated with rice roots from the different crop stages and rotations. According to multivariate, clustering and statistical analyses performed on results retrieved by *nifH* T-RFLP (primers F2/R6 and PolF/PolR and AluI), the interaction of root compartment and crop growth stage (PERMANOVA, $p < 0.001$) was the major driver of diazotrophic communities. Furthermore, a significant effect of the rice rotation systems on the structure of diazotrophic communities was found (PERMANOVA, $p < 0.05$), suggesting that crop intensification could impact diazotrophic communities associated with rice plants, which play a key role in plant growth promotion. The implications that this could have should be explored and considered when developing sustainable intensification strategies in rice production.

Keywords Rice rotation systems · Endophytes · Rhizospheric bacteria · Diazotrophs · *nifH* gene · Terminal restriction fragment length polymorphism

Introduction

Rice is a staple crop particularly relevant to the ever-growing world population. Therefore, a necessary increase in sustainable productivity through the intensification of land use remains a significant challenge. Biological nitrogen fixation (FBN) is a well-known fundamental process in nature that greatly contributes to the sustainability of agricultural systems. Diverse prokaryotes belonging to *Bacteria* and *Archaea* domains can transform molecular nitrogen into ammonia (Martinez-Romero 2006). The contribution of the free-living and non-symbiotic nitrogen-fixers and the role of a diverse range of aerobic and anaerobic organisms to this process were recently recognized (Ladha et al. 2016). However, the diversity of diazotrophs in complex environments like soils has been poorly addressed. Furthermore, the physiology and phylogeny of many diazotrophic microorganisms remain unknown according to analyses of *nifH* sequences (Gaby and Buckley 2011).

Root-associated microorganisms, particularly endophytes, play vital roles in plants' health, nutrition, growth, and coping with stress factors (Sessitsch et al. 2012; Hardoim et al.

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2015; Etalo et al. 2018; Compant et al. 2019). Besides the well-known association between *Rhizobium* and legumes, diazotrophic microorganisms have also been found to be associated with cereals as endophytes (Olivares et al. 1996; Baldani et al. 1997; Rosenblueth et al. 2018). Diazotrophic communities associated with cereals have been extensively studied by culture-independent methodologies mainly based on the marker gene of nitrogen fixation, i.e., *nifH* gene (Prakamhang et al. 2009; Hardoim et al. 2015; Rosenblueth et al. 2018) and indirectly by metagenomic approaches (Sessitsch et al. 2012). Different factors have been described affecting the diversity, structure, and activity of diazotrophic communities associated with rice roots, including the rice variety or landrace (Knauth et al. 2005; Elbeltagy and Ando 2008; Rangjaroen et al. 2014) and agricultural practices such as the use of N- fertilizers and irrigation practices (Wartiainen et al. 2008; Prakamhang et al. 2009; Zhang et al. 2021), among others. Oxygen availability is one of the main drivers of the structure and composition of microbial communities and a major factor in the evolutionary force determining the metabolic strategies of bacteria colonizing an environmental niche (Voroney 2007). Irrigation management not only have been shown to impact on diversity and structure of soil (Breidenbach and Conrad 2014) and endophytic microbiomes (Hardoim et al. 2012; Vishwakarma and Dubey 2020) but also have caused sharp shifts in endophytic diazotrophic communities associated with rice roots (Ferrando and Fernández Scavino 2015).

In Uruguay, rice is directly drilled in dry soil with minimum tillage or no-till, remaining under upland conditions until the V4–V5 growth stage (tillering). The rice crop is flooded at this stage, remaining in this condition until ten days before harvest, constituting a peculiar irrigation management compared to other rice production systems. The traditional Uruguayan rice crop constitutes a unique low-intensive cropping system characterized by rotating annual rice crops with mixed pastures for grassing livestock that has been used for more than 50 years. Rice–pastures rotation provides sustainability advantages linked to better soil quality and reduced dependence on agrochemicals compared with other rice systems worldwide (Deambrosi 2003; Pittelkow and Krupnik 2016; Macedo et al. 2021b). In the last 50 years, Uruguay has continuously increased its rice productivity (Tseng et al. 2020; DIEA 2022) with relatively low to moderate N fertilizer input; 70–90 kg ha^{-1} contrasting to the 120 and 200 kg ha^{-1} used in South Asia and South America, respectively (Chauhan et al. 2017), explained by biological N fixation in pastures and crops and recycling of organic N by livestock (Castillo et al. 2021).

Despite the high and stable rice yields reached during the last years, the Uruguayan rice producers have intensified the soil use, i.e., by shortening the years of pastures or by incorporating other crops like soybean in order to improve their productivity. Several authors have reported a beneficial influence of crop rotation practices on diversity, abundance, and composition of microbial communities from soils and associated with plant roots compared to monocropping (Xuan et al. 2012; Fernandez-Gnecco et al. 2021; Ma et al. 2021; Fadiji et al. 2021). Moreover, these changes in soil and plant-associated microbiomes could also impact crop yields. For example, Neupane et al. (2021) unveiled an association between soil health indicators related to microbiomes and soybean yields. Additionally, an impact of crop rotation on other microbial guilds in paddy soils has been previously reported. For example, the structure and function of methanogenic populations from Uruguayan soils shifted between pasture and irrigated rice fields (Scavino et al. 2013), and rotating flooded rice with upland maize impacted the resident and active community of this environmentally relevant microbial guild (Breidenbach et al. 2017).

There is a lack of knowledge about the impact of these intensification practices on diazotrophic communities associated with rice plants, which are vital for the present and future rice crop sustainability, especially in Uruguayan rice cropping systems. This work focused on studying endophytic and rhizospheric diazotrophic communities from rice roots from three contrasting rice rotation systems (rice–pastures, rice-soybean, and continuous rice) from a long-term field experiment. In addition, two different crop growth stages were studied, i.e., tillering before flooding the field and flowering at flooded conditions. The objectives were (1) to determine the abundance and diversity of rhizospheric and endophytic diazotrophic communities associated with rice roots from the three contrasting rice rotation systems at two crop growth stages (2) to compare the diazotrophic diversity associated with rice roots retrieved by using two different primer sets targeting *nifH* gene (PolF/PolR and F2/R6), and (3) to evaluate the influence of the cropping systems and crop growth stage on the diversity and community structure of diazotrophic communities associated with rice roots.

Materials and methods

Field experiment and sample collection

A field-scale experiment was installed in 2012 at Paso de la Laguna, Experimental Station of the National Agricultural Research Institute (INIA), in Treinta y Tres, Uruguay (33°16′23″ S; 54°10′24″ W; 22 MASL). The site has a mesothermic humid climate with a monthly mean annual temperature of 22.3 and 11.5 °C during summer and winter, respectively. Mean annual precipitation is 1360 ± 315 mm, similarly distributed during the year. According to USDA Soil Taxonomy (Durán et al. 2006), the dominant soil is an Argialboll with a silty clay loam texture (18% sand, 52% silt,

30% clay) and a slope less than 0.5%. Soil physicochemical properties, when the experiment was installed were following: TOC 14.2 g kg^{-1}, TN 1.4 g kg^{-1}, P Bray 7.0 µg g^{-1} and pH 5.7. Previously, the soil at the site was under a rice–pastures rotation system (approximately 2 years of rice and 3 years of pastures) for 34 years.

The long-term experiment evaluates six rice rotation systems under no-till with contrasting soil use intensity determined by the proportion of rice, pastures, and other crops in the rotation (Macedo et al. 2021a). The experiment was arranged in a randomized complete block design with three replications and all phases of the rotations (crop and pastures) simultaneously present (Patterson 1964), totalizing 60 plots of 20 × 60 m. Three contrasting rotation systems were selected for this work (see the scheme of the rotation systems selected in Fig. S1), each one in a single rice phase (R): (a) continuous rice (*Oryza sativa*) (rrrR: rice every summer); (b) the first rice after a pasture from a rice–pasture rotation, pppR: 2 consecutive rice crops followed by 3.5 years of a mixed pasture of tall fescue (*Festuca arundinacea*), white clover (*Trifolium repens*) and birdsfoot trefoil (*Lotus corniculatus*); and (c) rice after two previous years of soybean (*Glycine max*) and one year of rice (rssR: first year of rice followed by two consecutive years of soybean, a second year of rice and 2.5 years of a mixed pasture of *Festulolium* spp. and birdsfoot trefoil). In all rotations, ryegrass (*Lolium multiflorum*) and/ or *Trifolium alexandrinum* L. were used as cover crops during fall-winter between cash crops (Macedo et al. 2021a). All leguminous plants employed in the experiment, i.e., soybean, white clover, and birdsfoot trefoil used in rotation with rice and cover crops in winter, were inoculated with BIOFORCE® (Calister), a commercial bioinoculant based on *Bradyrhizobium japonicum,* following the manufacturer's recommendations.

Rice was no-till drill seeded in the last week of October 2015, emerged on November 10th, and flooded approximately four weeks after emergence at rice V4–V5 growth stages. Nitrogen (N) fertilizer was applied as urea in two growing stages, at V4 tillering (dry soil) and immediately before panicle initiation R0 (flooded soil). Annual nitrogen rates were 80–100 kg ha^{-1} of N in rice of pppR and rssR, while in rrrR was 160 kg ha^{-1} of N, following national guidelines and recommendations (Macedo et al. 2021a). Further information regarding the field experiment, including management practices, were as reported by Macedo et al. (2021a). The sampling was performed in the 2015–2016 growing season, four years after the experiment was set up. Bulk soils and rice plants were collected from each of the three blocks (replicates) of each rotation system selected at two different phenological stages of rice growth, representing different water regimes. The first sampling was carried out in December 2015 at tillering, 30 days after emergence, just before flooding and N application (TBF, Tillering-Before Flooding), while the second was performed in February 2016 at flowering, 98 days after emergence and 67 days after flooding (FF, flowering-flooded). Five bulk soil cores (0–10 cm depth) were randomly taken from each plot using an auger for coring between lines, homogenized by hand, and composited into a single sample from each replicate. $N–NH_4^+$, $N–NO_3^-$, redox potential, and pH of soils at the different growth stages were previously determined and reported (Fernández-Scavino et al. 2022; Martínez-Pereyra 2020) and are summarized in Table S2. For each plot, five (in TBF) or three (in FF) rice plants were randomly collected, containing the soil surrounding the root, and brought back to the laboratory on ice, with the bulk soil samples, for their subsequent processing.

Sample processing and superficial disinfection of rice roots

Bulk soil samples (S) were sieved through a 6 mm mesh to remove larger impurities (stones and plant debris), air dried at 30 °C to constant weight, sieved again through a 3 mm mesh, and finally stored at room temperature for physicochemical analyses and at − 20 °C for molecular analysis. The soil loosely attached to the roots of each plant was manually removed. Suspensions of rhizospheric soil (Rh) were obtained under aseptic conditions by shaking vigorously the roots and soil tightly attached to them in 50 mL (in TBF) or 100 mL (in FF) of sterile distilled water, pooling three or five plants, depending on the crop growth stage, and obtaining a rhizospheric soil suspension for each plot. The suspensions were then centrifuged, the supernatants discarded, and the pellets with the rhizospheric soil samples were stored at − 70 °C for DNA extraction. The rice roots used for rhizospheric soil suspension were then separated from the leaves, washed with tap water, and superficially disinfected with 2% sodium hypochlorite, followed by four rinses with sterilized distilled water, according to Ferrando and Fernández Scavino (2015) Disinfected roots (R) were frozen in liquid nitrogen and kept at − 70 °C for further analyses.

DNA extraction materials

DNA was extracted from 0.5 g of bulk soil and rhizospheric soil samples using PowerSoil DNA Isolation Kit (MOBIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. Frozen surface-disinfected rice root samples were pulverized in sterilized pre-cooled mortars with liquid nitrogen, transferred to sterilized 2.0 mL microtubes, and immediately stored at − 70 °C. DNA was extracted from 0.2 g of pulverized roots according to (Doyle and Doyle 1987), as described by Ferrando and Fernández Scavino (2015). DNA was quantified with the Qubit™

dsDNA HS Assay Kit, using a Qubit® 2.0 Fluorometer (Invitrogen).

The abundance of diazotrophs by qPCR of *nifH* genes

The abundance of *the nifH* gene was determined by Real-Time PCR (qPCR) using a Rotor-Gene® 6000, model 5-Plex (CORBETT Research, Sidney) as described by Ferrando and Fernández Scavino (2015). The reaction mixture contained 1 µL of diluted (one tenfold) template DNA, 1 µM of primer PolF and PolR (Table S1), and 5 µL of Rotor-Gene SYBR Green PCR Mastermix (QIAGEN®, Hilden, Germany). The thermal cycle was as follows: an initial step at 95 °C for 5 min followed by 35 cycles of 35 °C for 5 s and 60 °C for 10 s. The fluorescence signal was measured once per cycle after the annealing-elongation step by adding one step at 82 °C for 1 s. The program ended with a melt curve from 65 to 94 °C. All samples were amplified in duplicate, and the standard curves were generated for each qPCR run in triplicate for each standard point (ten-fold dilutions from 10^{-5} to 10^{-10}). Triplicates of no-template controls were included in each run as a negative control. The results were expressed per ng of total DNA extracted to avoid the bias in extracting DNA from different materials, presenting different extraction efficiencies. The data from *nifH* gene abundance were $\log_{10}$ transformed to generate a normal distribution of residues and homogeneity of variance. Statistical analysis was performed using R software version 4.1.1 (R Core Team, 2021), and a two-way ANOVA with Tukey's multiple comparisons test was used to compare the *nifH* abundances.

Terminal restriction fragment length polymorphism (T-RFLP) of *nifH* gene and AluI

The structure of diazotrophic communities was determined through T-RFLP analysis using two different primers sets, i.e., PolF/PolR and F2/R6 (Table S1). Both primers' sets bind to the same position along the reference *nifH* gene sequence of *Azotobacter vinelandii* (Genbank ACCN# M20568), with slight differences in degeneracy and oligonucleotide length. All PCR reactions were performed in a 25 µL reaction mixtures containing 10 × Taq buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 mM and 1 mM of each primer for PolF/PolR and F2/R6 respectively with the forward primers labeled with 6-carboxy-fluorescein, 0.2 mg mL^{-1} bovine serum albumin (Roche®), 1 U of Taq DNA polymerase (Fermentas©) and 1 µL of template DNA. The reactions were performed in a thermocycler (Applied Biosystems, 2720 Thermal Cycler, Singapur), and the PCR conditions are shown in Table S1. Three technical replicates were performed and pooled after PCR amplification and checked for amplification specificity on a 1.5% agarose gel. The PCR products were concentrated and desalted using the DNA Clean & Concentrator™ (ZYMO Research, USA). Aliquots containing approximately 150–200 ng of labeled PCR products were digested with AluI at 37 °C overnight. Restriction fragments were analyzed in Macrogen Inc. (Seoul, Republic of Korea) using an ABI 3730XL DNA Analyzer (Applied Biosystems). The size of each terminal restriction fragment (T-RF) was determined by comparison to a fluorescently labeled size standard (GeneScan TM 500 LIZ TM) using the Peak Scanner TM Software v1.0 (Applied Biosystems). Data exported from Peak Scanner software were processed with T-REX, an online software for the analysis of T-RFLP data (http://trex.biohpc.org/; Culman et al. 2009). TRFs under 50 bp and above 360 bp in size were deleted, noisy peaks were filtered from true peaks using a standard deviation of 1.0, and peaks were aligned with a clustering threshold of 1.0 bp. The relative abundance within each sample was calculated. Venn diagrams were generated using the online software Evenn (http://www.ehbio.com/test/venn/; Chen et al. 2021) to visualize the shared and unique T-RFs among the three crop rotation systems.

Diversity indices (Chao1, Shannon, and Simpson) were calculated for each sample from datasets retrieved with both primer sets separately using the function 'estimate_richness' in the Phyloseq package using R software version 4.1.1 (R Core Team, 2021). Statistical analyses also were performed using R software and a one-way ANOVA with Tukey's multiple comparisons tests. A PERMANOVA based on Bray–Curtis distance was performed to compare diazotrophic community composition between compartments (Rh, R) and crop rotation system at TBF and FF for the whole data set, i.e., T-RFs retrieved with both primer sets. In addition, non-metric multidimensional scaling (NMDS) ordination of T-RFLP of *the nifH* gene with the two primers sets was performed. Additionally, Clustering analysis was performed based on Bray–Curtis distances generated from T-RFLP profiles using Ward's method for the dataset retrieved with each primer set. A heatmap of the Mean Relative Abundance (MRA) of each T-RF for the two primers' datasets was constructed in Excel®. Principal Component Analyses (PCA) were performed, and variable vector plots were obtained per crop stage for the two combined datasets using FactoMineR and ggplot2 packages in R software.

T-RFs identification based on *nifH* gene cloning and 454 pyrosequencing

The identification of those dominant endophytic T-RFs that were detected in both datasets (PolF/PolR and F2/F6) was inferred from root endophytic *nifH* sequences retrieved in a previous work on rice plants grown on paddy soils from the same Experimental Station under greenhouse conditions (Ferrando and Fernández Scavino 2015)

by cloning (GenBank nucleotide accession numbers: KF872844–KF872993) and 454-pyrosequencing of *nifH* gene (NCBI BioProject number PRJNA885822) using primers PolF/PolR. An in silico analysis of both *nifH* libraries allowed us to determine the theoretical T-RFs generated by each *nifH* sequence. The sequences with theoretical T-RF matching those endophytic T-RFs obtained in this work (± 2 bp) were compared online to the protein database from GeneBank using the tool BlastX (https://blast.ncbi.nlm.nih.gov/Blast.cgi) in order to identify them. The closest NifH protein sequences with the highest identity percentage were reported.

Results

Abundance of diazotrophs by *nifH* qPCR

The abundance of the *nifH* gene was retrieved with primers PolF/PolR from bulk soil, rhizospheric soil, and rice roots from different rice rotation systems at two different crop growth stages. A significant interaction was found between the crop stage and plant compartment (Table S3). There were significant differences in *nifH* gene abundances between the TBF and FF stages for rice roots and rhizospheric soils (Table 1 and Table S3, p value = 0.00105). At the same time, there were no significant differences for those from bulk soils. Additionally, the rhizospheric soils possessed the highest *nifH* copies per g of DNA at the FF stage, whereas the TBF rice roots had the lowest abundance of diazotrophs according to *nifH* qPCR. Conversely, the abundance of diazotrophs in the different compartments of the three rice rotation systems studied did not show significant differences in any of the compartments or crop growth stages (Table 1 and Table S3).

Diversity of rhizospheric and endophytic diazotrophs associated with rice roots by T-RFLP of *nifH* gene

The T-RFLP profiles for the *nifH* gene using AluI restriction enzyme retrieved from rhizospheric soils and rice roots of the three rice rotation systems at two different crop growth stages are shown as mean relative abundances (MRA, n = 3) of the dominant T-RFs retrieved (MRA > 1%) for F2/R6 and PolF/PolR primers' sets in Figs. 1 and 2 respectively. After the processing, standardization, and analysis with online T-REX software, a similar number of T-RF was retrieved for the primer sets PolF/PolR and F2/R6 (30 T-RFs and 34 T-RFs, respectively). Twenty-six T-RFs were common to both primer sets, while 4 and 8T-RFs were exclusively retrieved by PolF/PolR and F2/R6 primers.

Diversity indices were calculated from T-RFLP profiles from each primer set (Fig. 3). Both primer sets showed differences in the diversity of diazotrophic communities associated with rice roots in the different crop growth stages according to the statistical analyses performed (Fig. 3). The statistical comparison of Chao1 indices showed that a higher diversity was retrieved in rhizospheric and endophytic diazotrophic communities from the FF stage compared to those from TBF stage (Chao1 index; PolF/PolR: p value < 0.001; F2/R6: p value = 0.00216). Shannon indices showed different results for the two datasets. A significant interaction was detected between the crop growth stage and compartment (p value < 0.001) and between the crop growth stage and rotation system (p value = 0.00567) for primers PolF/PolR. The highest Shannon indices corresponded to rice roots from the FF stage, significantly different from those retrieved from rice roots at the TBF stage. In addition, diazotrophic communities from rssR-rhizospheric soils at the TBF stage had lower diversity than those from pppR and rrrR rotation systems, according to the Shannon and Simpson indices retrieved with primers PolF/PolR (Fig. 3).

When Shannon indices retrieved from F2/R6 dataset for different stages and plant compartments were compared, significant differences in diazotrophic diversity were only observed for rice roots (FF rice roots > TBF rice roots; p

Table 1 Abundance of *nifH* genes in rice roots, bulk, and rhizospheric soils from different crop rotation systems at two rice crop growth stages[a]

Rotation	Log_{10} *nifH* gene copies. ng of DNA^{-1}					
	Rice roots		Rhizospheric soil		Bulk soil	
	TBF d	FF c	TBF b	FF a	TBF c	FF c
rrrR	2.24 ± 0.46	3.06 ± 0.20	3.61 ± 0.19	4.65 ± 0.51	3.02 ± 0.06	2.80 ± 0.58
pppR	2.25 ± 0.52	2.93 ± 0.41	3.55 ± 0.26	4.34 ± 0.25	2.88 ± 0.24	2.92 ± 0.14
rssR	2.26 ± 0.09	3.01 ± 0.10	3.39 ± 0.17	3.61 ± 0.65	3.05 ± 0.07	2.98 ± 0.21

[a]The abundance of *nifH* genes retrieved with primers PolF/PolR are expressed as mean ± standard deviation (n = 3). Different letters in columns indicate significant differences (p value < 0.05) based on Tukey's HSD test

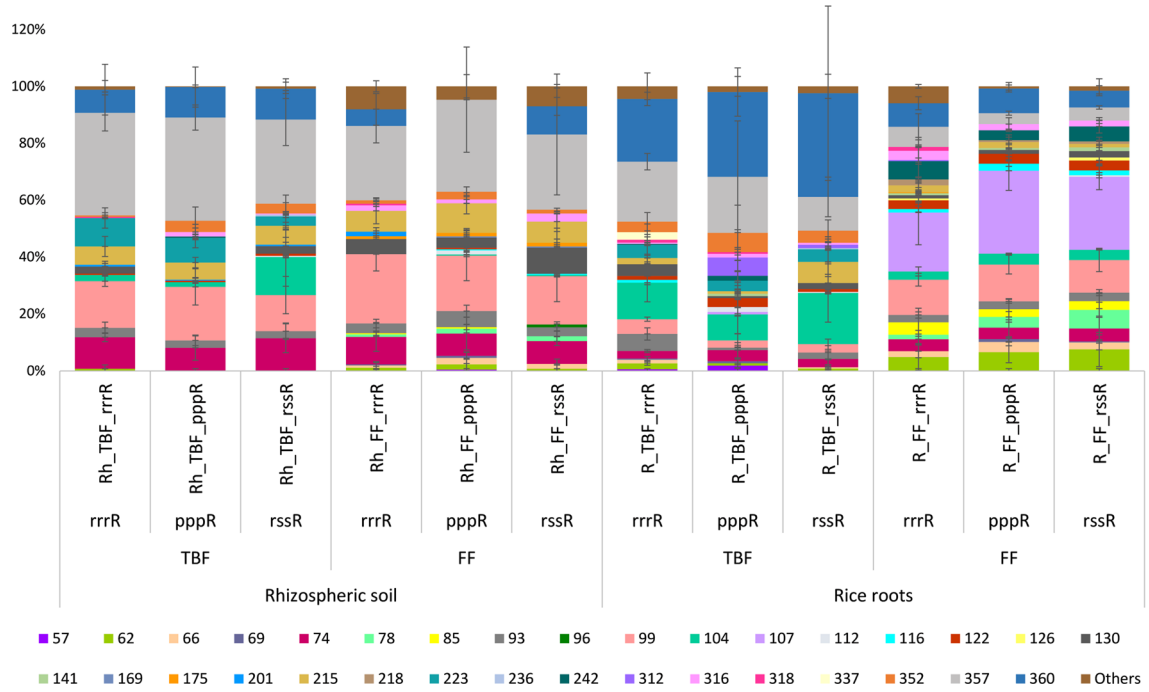

Fig. 1 T-RFLP profiles of *nifH* gene retrieved with primers F2/R6 and AluI restriction enzyme from rhizospheric soil and rice roots before flooding (TBF) and after flooding (FF) under different rotation systems (rrrR: continuous rice, pppR: rice–pastures, rssR: rice-soybean). Different color bars correspond to different T-RF sizes indicated in the legend in base pairs (bp). Mean relative abundances (n = 3) are shown, and standard deviations are included for each bar. Others: T-RFs with less than 1% of relative abundance

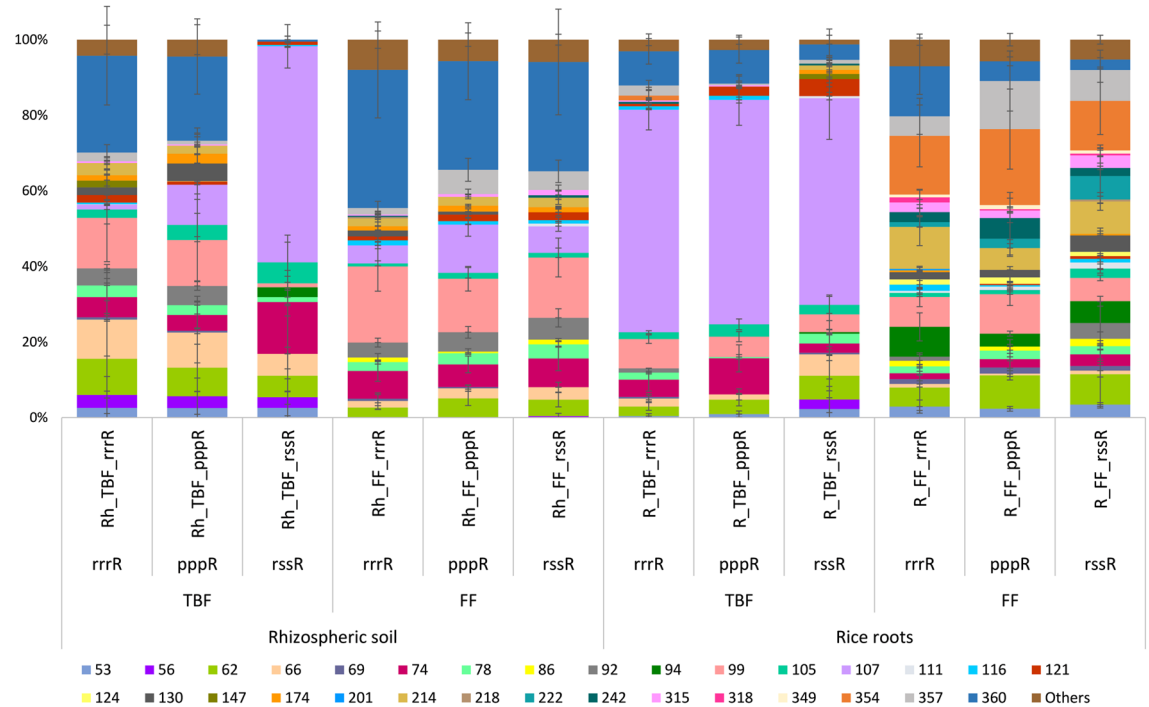

Fig. 2 T-RFLP profiles of *nifH* gene retrieved with primers PolF/PolR and AluI restriction enzyme from rhizospheric soil and rice roots before flooding (TBF) and after flooding (FF) under different rotation systems (rrrR: continuous rice, pppR: rice–pastures, rssR: rice-soybean). Different color bars correspond to different T-RF sizes indicated in the legend in base pairs (bp). Mean relative abundances (n = 3) are shown, and standard deviations are included for each bar. Others: T-RFs with less than 1% of relative abundance

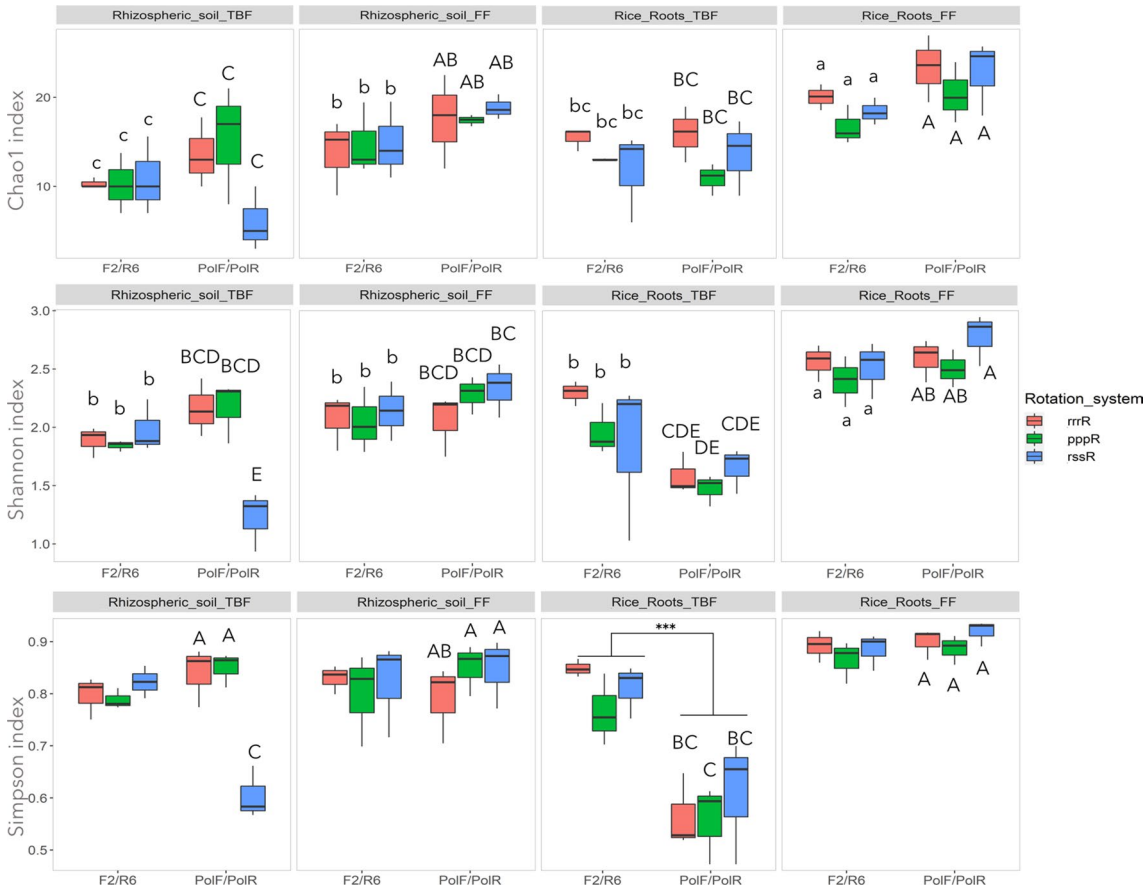

Fig. 3 Alpha diversity indices (Chao1, Shannon, and Simpson) for T-RFLP profiles of *nifH* gene retrieved with primers F2/R6 and PolF/PolR (n = 3). Different letters, lowercases for F2/R6 dataset and uppercases for PolF/PolR dataset, indicate significant differences for each index within each dataset (ANOVA and Tukey's test $p < 0.05$). Significant differences between primer sets for each index are shown with asterisks

value = 0.00216). In addition, the Simpson index for F2/R6 dataset did not show significant differences by any factor. In contrast, a significant interaction among the compartment, growth stage, and rotation system was observed for the Simpson index of the PolF/PolR dataset (p value = 0.0073). TBF rhizospheric soils from the rssR rotation system had the lowest Simpson index and significantly differed from those from the pppR and rrrR rotation systems. When comparing the results obtained with the two primer sets, it was observed that diazotrophic diversity in TBF rice roots, retrieved with primers F2/R6, was significantly greater than with PolF/PolR according to the statistical comparison of Simpson index for both datasets (p value < 0.001, Fig. 3), whereas Chao1 and Shannon's indices did not show any significant differences between primer sets.

On the other hand, beta diversity analyses were performed to compare the diazotrophic communities from rice roots and rhizospheric soils from the different crop rotation systems and stages. NMDS, clustering, and PERMANOVA analyses were performed from the whole dataset, i.e., all T-RFs and their relative abundance obtained from the two primers' sets and restriction with AluI. NMDS ordination analysis (Fig. 4; stress = 0.1159975) showed that diazotrophic communities mainly grouped according to the compartment (Rhizospheric soil and rice roots) and crop stage (TBF and FF). PERMANOVA analysis confirmed this result, which showed that the interaction between the compartment and crop stage was significant ($R^2 = 0.13$, p value = 0.0001). The crop rotation system also affected the clustering of diazotrophic communities ($R^2 = 0.0468$, p value = 0.0355), as is shown in Table S4.

Additionally, clustering analyses for the *nifH* T-RFLP datasets obtained with both primers sets and based on Ward's distance showed that diazotrophic communities grouped mostly by crop growth stage (Fig. S2). The compartment also showed to influence the grouping of diazotrophic communities. Rhizospheric and root endophytic diazotrophic communities from the FF stage comprised a cluster. Within this cluster, roots' T-RFLP profiles were highly similar (about 75% similarity) and grouped in a distinct subcluster.

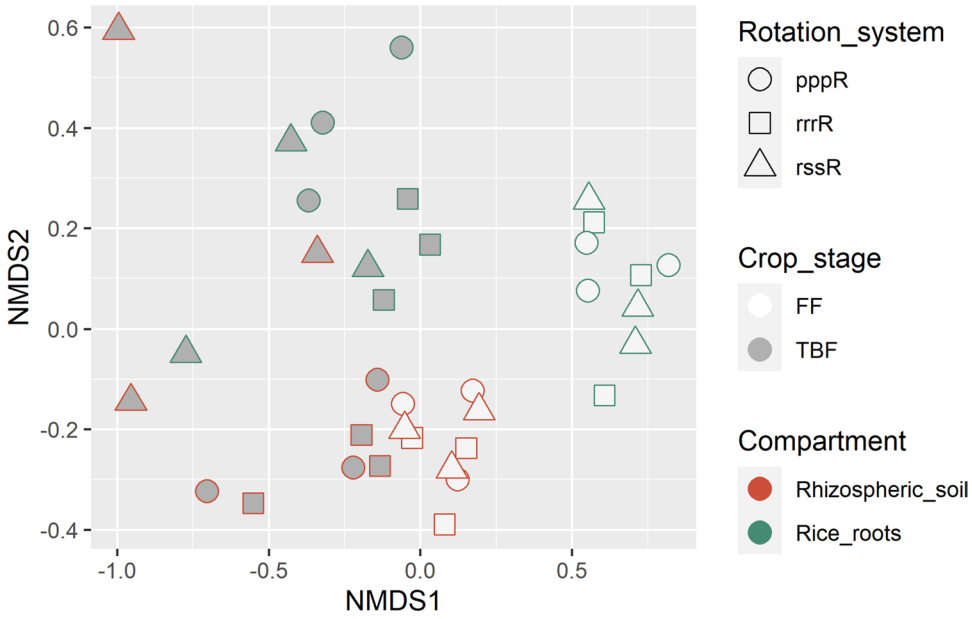

Fig. 4 Bray–Curtis-based Non-Metric Multidimensional Scaling (NMDS) ordination of *nifH* gene T-RFLP profiles retrieved from the whole dataset (both primers sets and AluI restriction enzyme) from rhizospheric soils and rice roots at tillering before flooding (TBF) and flowering flooded (FF) stages (n = 3). Stress value = 0.1159975. 9999 permutations

Conversely, diazotrophic communities from TBF rice roots clustered mainly with those from TBF rhizospheric soils. These results were consistent with those obtained with NMDS and PERMANOVA.

PCA analyses by crop stage were also performed on *nifH* T-RFLP data from both datasets. The contribution of the dominant T-RFs (MRA > 1%) to the clustering of diazotrophic communities was evaluated (Fig. 5). At the TBF stage, diazotrophic communities from rice roots and rhizospheric soils were grouped with a data variability explained of 31.4% (Fig. 5a). Diazotrophic communities associated with rice roots were clustered by compartment except for the rssR rotation where rhizospheric soils and rice roots were distributed in between the observed compartment clusters. For this rice rotation, the distribution of the diazotrophic communities from both compartments overlapped. The endophytic diazotrophic communities from the rrrR and pppR rotations were tightly grouped within each treatment, and those from the rssR rotation were grouped separately from the rrrR rice roots. The 30 T-RFs found as the major clustering contributors are shown in Fig. 5b. The T-RF 107 bp (PolF/PolR dataset) was the most relevant in grouping endophytic diazotrophic communities at the TBF stage, followed by 104–105 and 360 bp (F2/R6 dataset) and 121–122 bp (PolF/PolR dataset). The grouping of rrrR diazotrophic communities, clustered separately from the other rotation systems, was mainly influenced by the T-RF 318 bp (F2/R6 dataset) and 357 bp (PolF/PolR dataset). Conversely, at this crop stage, rhizospheric communities were not grouped by rotation systems, and a more dispersed distribution of diazotrophic communities was observed (Fig. 5a). Several T-RFs from both datasets contributed to the distribution of diazotrophic rhizospheric communities. The rhizospheric diazotrophic communities from the rssR rotation system were affected by other T-RFs that also influenced the grouping of endophytic communities.

On the other hand, at the FF stage, rhizospheric soils and rice roots diazotrophic communities were distinctly clustered (Fig. 5b), and the PCA analysis explained 43% of data variability. In this case, the crop rotation systems did not affect the clustering of endophytic or rhizospheric diazotrophic communities. The T-RFs 74, 107, 360, 174, 66 bp (PolF/PolR dataset) and the 214–215, 99, 92–94, 357, 352 bp (F2/R6 dataset) were the principal T-RFs responsible for the grouping of rhizospheric soils (Fig. 5b). In addition, the gathering of endophytic diazotrophic communities at the FF stage was mainly influenced by T-RFs 104, 107, 62, 121–122, 242, 85 bp (F2/R6 dataset) and the T-RFs 53,214–215, 354, 86 bp (PolF/PolR dataset) (Fig. 5b).

Dominant T-RFs in rhizospheric and endophytic diazotrophic communities and identification of dominant endophytic T-RFs

The T-RFLP datasets retrieved with both primers' sets shared more than 70% of T-RFs (Fig. S3), accounting for 31–86% of the total MRA. The MRA of some shared T-RFs varied with the primer set used. Unique T-RFs detected in each dataset presented MRA < 2.5%.

The distribution of each T-RF across the different compartments, crop stages, and primers' sets is shown in the heatmap of MRA (Fig. S3). The fragments corresponding to unrestricted *nifH* amplicons (357 and 360 bp) were detected with both primers' sets in the two compartments in all crop stages and rotation systems but dominated in the rhizospheric diazotrophic communities accounting for a total

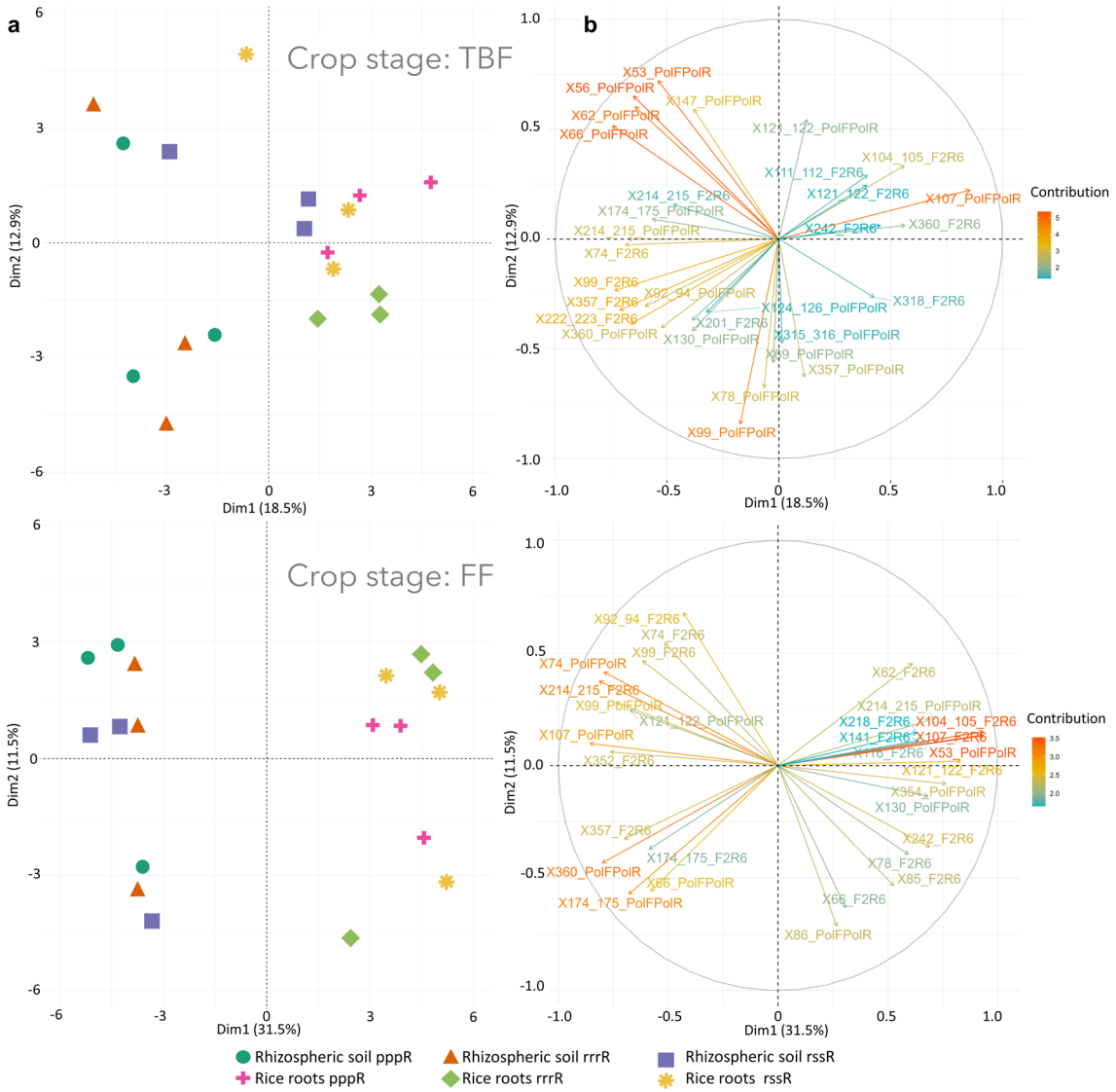

Fig. 5 Principal component analysis (PCA) of *nifH* T-RFLP profiles (**a**) and contribution of T-RFs to the clustering of diazotrophic communities (**b**) by primers' set (PolF/PolR and F2R6) and crop stage (TBF and FF). T-RF sizes (bp) are preceded by an X. Vectors correspond to the Individual scores of the T-RFs on clustering. Color gradient and vector length indicate the T-RF contribution

MRA (357 plus 360 bp) between 17 and 43%. The amplicons without restriction site were also present in endophytic diazotrophic populations with lower MRA in most rice roots (8.6–15.6%) except for TBF rice roots retrieved with primers F2/R6, which reached 45%. Other dominant T-RFs were also widely distributed regardless of the compartment, crop stage, or primers used (62, 74, 93, 99, 104, 112, 130, 215, 316 bp), which represented a mean of 46.8 and 36.8% of the total MRA retrieved with primers F2/R6 and PolF/PolR, respectively, in rhizospheric soils. Whereas inside rice roots, these shared dominant T-RFs accounted for 64.6 and 41% of the total MRA, respectively.

A similar alpha diversity retrieved from *nifH* T-RFLP profiles from rice roots was observed when previous (Ferrando and Fernández Scavino 2015) and present work were compared (data not shown). Additionally, no major differences in alpha diversity were obtained between the two datasets in the present study (Fig. 3). Thus, according to these considerations and those described in M&M section, the identity inference was made only for dominant T-RFs from endophytic communities detected in both datasets (Table S5; Ferrando and Fernández-Scavino 2015) using the previous *nifH* cloning and 454 pyrosequencing data. Conversely, dominant rhizospheric T-RFs could not be identified since no information was available.

The two primer sets detected twelve dominant T-RFs in all crop stages, root compartments, and rotation systems, although variation in MRA was observed (Fig. S3). The

T-RF 92–94 bp was dominant and ubiquitous in rhizospheric soils and rice root tissues regardless of crop stage and rotation system. Their presence as part of endophytic communities could be related to NifH sequences from the alphaproteobacterial *Herbaspirillum* spp. and *Pleomorphomonas* spp., and the firmicutes *Paenibacillus* sp. at the TBF stage (Table S5; 97.92, 96.19 and 100% identity, respectively), whereas at the FF stage it could be linked to several betaproteobacteria (*Ideonella*, *Variovorax*, *Hydrogenophaga*, among others, Table S5) and gammaproteobacterial genera/ species (*Kosakonia* spp., *Enterobacter* sp., among others).

The T-RFs 74 and 130 bp were abundant in all stages and compartments and particularly enriched in rhizospheric soils (MRA about 9, and 4%, respectively). Likewise, the T-RF 214–215 bp was broadly distributed, with higher MRA observed for rhizospheric soils (MRA 7.3%). The MRA of this T-RF was found to increase in rice roots from the rssR rotation system at the TBF stage (Fig. S3; MRA 7.2%). None of the *nifH* sequences from the previous work retrieved an *in-silico* T-RF of this size from rice roots under upland conditions. However, under flooded conditions, several *nifH* clones and sequences belonging to phylogenetically and metabolically diverse species (*B. japonicum*, *Treponema azotonutricium*, *Desulfovibrio*-related spp., and verrucomicrobial bacteria; Table S5) generated a T-RF 214–215 bp.

The composition of diazotrophic endophytic communities inhabiting rice roots differed at the two crop growth stages according to T-RFLP profiles obtained (Figs. 1, 2). The rice roots had many T-RFs enriched at the two crop growth stages. The T-RF 104–105 bp was one of the most abundant and, as endophytic, was found poorly related to NifH sequences related to *Arcticibacter svalbardensis* (89.52% identity) detected under flooded conditions in previous work. However, this T-RF was enriched inside rice roots at the TBF stage (MRA 13% vs. 3.4% at the FF stage) and in rhizospheric soils at the same crop stage (Fig. S4).

On the other hand, at the FF stage, several T-RFs were enriched. The T-RF 107 bp was dominant inside rice roots and greatly enriched at the FF stage (MRA 25 vs. 0.27% at the TBF stage). This T-RF was found to be related to NifH proteins belonging to species of the alphaproteobacteria *Komagataeibacter* (Table S5, 91.43% Identity). Different distribution patterns were observed for this T-RF between the two datasets constituting a significant discrepancy between the MRA retrieved by the two primer sets (Fig. S3). The T-RFs 99 bp and 62 bp were also enriched inside rice roots at the FF stage (12.34 and 6.3%, respectively). The former was also abundant in rhizospheric soils regardless of the crop stage (MRA about 18%). Endophytic T-RF 99 bp could be related to NifH proteins belonging to *Pleomorphomonas koreensis* and *Kosakonia oryzae* (Table S5, 100, and 99.16%, respectively), whereas T-RF 62 bp could be related to NifH sequences from *Sulfurospirillum* species (Table S5; 98.25% identity). In addition, T-RFs 78 bp, related to NifH proteins belonging to *Rhizomicrobium electricum* (Table S5, 100% identity), was exclusively detected in the FF stage with MRA of 4% inside rice roots. Other T-RFs enriched in FF rice roots were 316 and 242 bp accounting for 3.3 and 5% MRA, respectively. The latter fragment was related to NifH sequences related to the betaproteobacterial species (*Ulginosibacterium gangwonense*, *Gallionella* sp.; > 99.17 and 100% identity, respectively). The T-RF 85–86 bp was abundant in the FF stage, linked to NifH protein sequences related to *Humidesulfovibrio mexicanus* or *Desulfovibrio* sp. UIB00 (Class *Deltaproteobacteria*; 97.2 and 94.23% identity, respectively) and *Hungatella xylanolytica* or *Clostridium indicum* (*Firmicutes*, 96.04% identity) were detected in roots and rhizospheric soils at FF stage with MRA about 1.5 and 1%, respectively (Fig. S3). The T-RF 242 bp was greatly enriched in FF rice roots (MRA 3.4–5%) and related to betaproteobacterial sequences (*U. gangwonense* 99.17% and 100% Identity; *Gallionella* sp. 100%).

According to Venn diagrams (Fig. S4), 15 and 18 T-RFs were shared among diazotrophic communities of rhizospheric soils and rice roots from the different crop rotation systems, respectively. Conversely, several T-RFs were associated with only one cropping system (Fig. S4). In TBF rhizospheric soils, the T-RFs 124–126, 236, and 147 bp were found exclusively in pppR, rssR, and rrrR, respectively. Whereas, in FF rhizospheric soils, the T-RFs 96 and 218 bp were found to be associated with rssR and rrrR, respectively. Additionally, endophytic *nifH* sequences related to *Cupidesulfovibrio* and *Ferriphaselus* (236 bp T-RF) were also associated with rssR rotation at the TBF stage, along with 147 bp T-RF that could not be identified. Conversely, rice roots from rrrR rotation at TBF and FF stages had T-RFs 337 and 201 bp associated with NifH sequences not identified and mainly related to *Desulfovibrio* spp., respectively (Table S5).

Discussion

The present work addressed the study of diazotrophic bacterial communities from paddy soils and associated (rhizospheric and endophytic) with rice roots from different rice rotation systems from a field experiment at two crop growth stages. In contrast to other reports, this work studied a stable and sustainable rice–pasture rotation system used for more than 30 years and its transition toward more intensive rice rotation alternatives. Thus, this work contributes to the knowledge of the impact of these agricultural practices on microbial guilds of great relevance, the nitrogen-fixing communities associated with plants, a subject scarcely addressed.

This work determined the abundance of *nifH* genes in soils, rhizosphere, and rice roots using the primers PolF/PolR, whereas for the diversity analyses of diazotrophic communities associated with rice roots, T-RFLP was performed using the primers' sets PolF/PolR and F2/R6. The performance of the several designed primers, not only on the diversity of *nifH* sequences retrieved (Gaby and Buckley 2012; Angel et al. 2018) but also on the abundance of *nifH* gene copies retrieved by qPCR (Gaby and Buckley 2017), have been evaluated by other authors. The primers PolF/PolR have been the most extensively used, and the studies above-mentioned have reported them as the least biased for *nifH* gene quantification (Gaby and Buckley 2017). However, they have low diversity coverage for diversity studies in environmental samples according to in silico analyses (Gaby and Buckley 2012). Conversely, the primers F2/R6 (Marusina et al. 2001) have been reported with one of the best in silico performances in the global *nifH* primer evaluation for diversity studies performed by Gaby and Buckley (2012). The capability of these and other primers' sets for recovering the *nifH* diversity in environmental samples was studied by Angel et al. 2018 using *nifH* Illumina MiSeq sequencing (primers PolF/PolR not included). These authors have shown that primers F2/R6 have a good performance in environmental samples, especially for roots and rhizosphere of rice, yielding higher richness and diversity estimates than other primers (PolF/PolR not included) for these compartments. Despite these reports, the F2/R6 primers have yet to be widely adopted for studies of diazotrophic diversity. There is a lack of information regarding comparison of the performance of primers F2/R6 vs. PolF/PolR under wet-lab conditions. Moreover, the latter primers have been probed to retrieve a wide variety of anaerobic endophytic diazotrophs from rice roots belonging to Cluster III (Zehr et al. 2003) by *nifH* cloning and 454-pyrosequencing according to the above-mentioned previous work.

Globally, in the present work, the *nifH* T-RFLP performed with primers F2/R6 and PolF/PolR did not show significant differences in diazotrophic diversity retrieved according to the diversity estimates obtained, except for the Simpson index for rice roots at the TBF stage for which greater values were observed for primers F2/R6. Additionally, both T-RFLP datasets shared more than 70% of T-RFs retrieved accounting for 31–86% of the total mean relative abundance. The primer sets presented different sensitivity to retrieve some *nifH* sequences, for instance, T-RFs 62, 104–105, 107, 223, and 352 bp, among others. All the several designed primers have shown different biases against the different diazotrophic phylogenetic clusters I–IV (Zehr et al. 2003; Raymond et al. 2004), even those reported with the best performances in silico (Angel et al. 2018), so using different *nifH* primer sets could allow to obtain a better picture of diazotrophic diversity in a particular environment.

On the other hand, in the Uruguayan rice crop (no-till direct-seeded), the rice plant remains in upland conditions until the mid-tillering stage when it is flooded. This sharp shift in the irrigation status causes changes in the environmental conditions of the soils, like redox and physicochemical properties (Kögel-Knabner et al. 2010). Our study, performed under field conditions, showed that the crop growth stage affected the abundance of *nifH* genes differently depending on the compartment studied. The abundance of diazotrophic populations in bulk soils was not affected by the crop growth stage of the rice plant, whereas diazotrophs associated with rice roots, not only rhizospheric but also endophytic, significantly increased their abundance at flowering under flooded conditions.

According to the T-RFLP analyses performed with both primer sets, the rhizospheric soils and rice roots sustained distinct diazotrophic bacterial communities. Rhizospheric and endophytic diazotrophic communities from rice roots significantly increased their diversity at the FF stage compared to the corresponding TBF stage, according to most of the diversity indices retrieved. In addition, the diazotrophic communities inhabiting rice roots at the FF stage were more diverse than those from rhizospheric soils at the same stage (Chao1 index, primers F2/R6). These findings reinforce the idea that the rice plant plays an active role in the assembly of endophytic diazotrophic communities. Furthermore, these results confirmed the strong impact of flooding on diazotrophic abundance and diversity observed for endophytic diazotrophic communities in a previous work under greenhouse conditions with soils from the same location and irrigation management (Ferrando and Fernández Scavino 2015). Additionally, unrestricted *nifH* amplicons were relevant in both datasets, particularly in rhizospheric soils. The relevance of this T-RF could represent a methodological limitation for studying diazotrophic communities associated with rice plants in these samples, especially in the TBF stage.

The identity of dominant endophytic T-RFs was inferred using data from diazotrophic endophytic community composition from previous work. According to the identification performed, metabolically and phylogenetically diverse diazotrophic communities were established in the inner tissues of disinfected rice roots from the different crop stages and rotation systems. Dominant T-RFs inhabiting rice roots belonged to *Proteobacteria* (Classes *Alpha*, *Beta*, *Gamma,* and *Delta* and *Epsilonproteobacteria*), followed by *Firmicutes*, *Bacteroidetes*, *Verrucomicrobia*, *Spirochaetes* and FCB group. Other authors have reported the same dominant phyla comprising rice root-associated microbiome and endophytic diazotrophic communities inhabiting rice roots (Sessitsch et al. 2012; Edwards et al. 2015; Rosenblueth et al. 2018; Ding et al. 2019). Most dominant T-RFs were related to *nifH* sequences detected in rice roots in a

previous greenhouse work by cloning and/or 454-pyrosequencing under flooded or unflooded conditions (Ferrando and Fernández Scavino 2015).

Fourteen T-RFs retrieved with both primer sets had MRA > 5% for at least one compartment, crop growth stage and/or crop rotation. Regarding endophytic diazotrophic communities, the unrestricted fragment dominated in TBF rice roots, whereas at the FF stage, the rice roots were mainly dominated by T-RFs 99 and 107 bp. The former T-RF could be closely related to NifH sequences of *P. koreensis* (Order Rhizobiales, closely related to *Pleomorphomonas oryzae*) isolated from rice roots and described by Xie and Yokota (2005) or a strain of *Kosakonia oryzae* (NCBI accession number ALH07171) retrieved from roots of the biofuel crop *Jatropha curcas*. The latter species and other *Kosakonia* spp. have been found as endophytes and PGP bacteria in rice (Mosquito et al. 2020), sugar cane, and groundnut (Taulé et al. 2019; Preyanga et al. 2021; Leite et al. 2021). *Komagataeibacter* spp., an acetobacteria closely related to the genus *Gluconacetobacter,* which includes well-known nitrogen-fixing species (Yamada et al. 2012) and is outstanding for its successful association with sugarcane (Cavalcante and Dobereiner 1988; Fischer et al. 2012), was also dominant inside rice roots, particularly enriched at FF stage (T-RF 107 bp). *Gluconoacetobacter* spp. were isolated as diazotrophs from rice roots of Indian cultivars by Jha et al. (2009). In addition, *Paenibacillus* spp. affiliated NifH sequences could be related to rice roots at the TBF stage (T-RFs 56–57 and 92–94 bp), coincidently to what was reported in a previous work (Ferrando and Fernández Scavino 2015). *Paenibacillus* was found to be associated as rice endophyte particularly relevant in rice cultivated under aerobic conditions (Vishwakarma and Dubey 2020), enriched in rice-tomato rotations in China (Ma et al. 2021), and isolated as diazotrophs from paddy soils (Islam et al. 2010).

Other genera, not so frequently reported as associated with plants, were also detected. Among them, *Sulfurospirillum* spp. (T-RF 62 bp) were among the dominant diazotrophs detected by T-RFLP inside rice roots at all stages. This genus was found fixing nitrogen actively in sugarcane roots (Burbano et al. 2011). While *nifH* sequences distantly related to the sphingobacteria *A. svalbardensis*, isolated from Arctic soils (Prasad et al. 2013), were enriched in the TBF stage, particularly inside rice roots (T-RF 104 bp).

Furthermore, the rhizospheric soils were mainly dominated by *nifH* sequences without the AluI restriction site (40–50% MRA). The T-RFs 62, 74, 99, 107, and 214–215 bp found inside rice roots were also dominant in rhizospheric soils. Data on the composition of rhizospheric diazotrophic communities associated with rice, at field or greenhouse conditions, were not available for taxonomical identification of the T-RFs retrieved, neither with PolF/PolR nor with F2/R6. Thus, further analyses based on *nifH* high throughput sequencing with primers F2/R6 should be performed to address the rhizospheric rice composition of this microbial guild in the different rotation systems and crop stages.

On the other hand, beta diversity analysis (NMDS and Clustering analysis) showed that diazotrophic communities grouped according to the compartment and crop growth stage. In addition, PERMANOVA analyses of the whole dataset showed a significant interaction between these two factors indicating that the developmental stage of the rice plant and the changes in irrigated conditions at the two sampling stages could not be analyzed separately at field conditions and are interrelated. Thus, these two factors combined were the main drivers of the diazotrophic communities associated with rice roots. Furthermore, other authors have shown that microbial communities from paddy fields are largely influenced by water regime not only in rice soil microbial communities (Breidenbach and Conrad 2014; Frindte et al. 2020) but also in rhizospheric and endophytic communities (Hardoim et al. 2012; Li et al. 2019; Vishwakarma and Dubey 2020). Moreover, diazotrophic communities from rice roots and rhizospheric soils clustered consistently and tightly by compartment at the FF stage, whereas more dispersed and variable diazotrophic communities were established at the TBF stage according to PCA analyses. A similar effect was observed in previous work under greenhouse conditions (Ferrando and Fernández Scavino 2015). Additionally, the T-RFs 107 bp (related to *Komagataeibacter* spp.) and 104–105 bp (poorly affiliated to the bacteriodetes *A. svalbardensis*) were the most relevant on clustering endophytic communities regardless of the crop stage. Whereas the T-RFs 360, 357, 318 bp (corresponding to unrestricted fragments and linked to unclassified *Ideonella*); and the T-RFs 53 (unidentified), 121–122 (related to Bacteroidetes–Verrucomicrobial *nifH* sequences), 62 (*Sulfospirillum* spp.), 214–215 (multiple assignations), 242 (*U. gangwonense* and *Gallionella* sp.) and 85 bp (*Humidesulfovibrio*, *Desulfovibrio*, and *Hungatella* related *nifH* sequences) contributed especially to the gathering of these endophytic communities at TBF and FF stages, respectively.

On the other hand, the impact of different rotation systems on diazotrophic communities associated with rice roots was studied. The crop rotation systems did not affect the abundance of diazotrophic communities from bulk and rhizospheric soils and rice roots. Globally, the diversity of diazotrophic communities was not affected by the rotation system used, except for rssR rhizospheric communities at the TBF stage, which had lower diversity indices Shannon and Simpson, retrieved from PolF/PolR dataset, than those from pppR and rrrR rotation systems.

Conversely and interestingly, the PERMANOVA analysis showed that crop rotation systems significantly affected diazotrophic populations inhabiting rice roots and rhizospheric soils ($p < 0.05$). Moreover, PCA results by crop stage

showed that the rotation systems did not affect the rhizospheric diazotrophic populations, regardless of the crop stage, whereas the endophytic diazotrophs were clustered by this factor. The endophytic diazotrophic communities from the rrrR and pppR rotations at TBF stage were tightly grouped within each rotation system, according to PCA analyses performed whereas the diazotrophic communities from the rssR rotation system were in a transitional zone between rhizospheric and endophytic diazotrophic communities from the other crop rotations. Interestingly, endophytic diazotrophic communities from continuous rice (rrrR) and rice–pastures rotation system (pppR) differentiated at TBF stage. These findings imply that the rotation systems and/or the previous pastures might be exerting an impact on diazotrophic endophytic communities established inside rice roots at the beginning of the following rice crop cycle. Thus, further studies should be conducted to elucidate this issue.

Additionally, although endophytic and rhizospheric diazotrophic communities from the different rice rotation systems shared 82 and 63% of total T-RFs, respectively, some T-RFs were found to be associated with the different cropping systems. For example, the T-RF 124–126 bp was exclusively detected associated with pppR rhizospheric soils at the TBF stage. Moreover, rice roots also showed *nifH* sequences related to the deltaproteobacterial and sulfate reducers *Desulfovibrio* spp. associated with pppR crop rotation at both crop growth stages and *Cupidesulfovibrio,* a recently described genus comprising several former *Desulfovibrio* species (Wan et al. 2021), was exclusively detected at the TBF stage in rice roots from rssR rotation. In addition, these anaerobic bacteria were found to play an active role in fixing atmospheric nitrogen under flooded conditions in paddy soils (Mårtensson et al. 2009) and inside rice roots (Knauth et al. 2005; Elbeltagy and Ando 2008), although in the present work they were also detected as root resident at tillering under aerobic conditions, as reported by Collavino et al. (2020) in their study on tomato endophytic diazotrophic community. Conversely, at the FF stage, unidentified rhizospheric T-RFs associated exclusively with rssR and rrrR were detected (96 and 218 bp, respectively).

In conclusion, the compartment and the crop growth stage were the main drivers of diazotrophic communities inhabiting the rhizosphere and roots of rice plants grown under field conditions, impacting their abundance, diversity, and composition. Although using two primers' sets did not significantly affect the diazotrophic diversity retrieved by *nifH* gene T-RFLP, it allowed us to better understand diazotrophic diversity inhabiting rhizospheric soils and rice roots. Under field conditions, a phylogenetically and metabolically diverse diazotrophic endophytic community was found inside rice roots. These communities included several well-known diazotrophs and other genera less studied but previously found as rice root endophytes that could play a relevant role, especially under such contrasting irrigation conditions experienced by rice plants throughout the crop cycle in Uruguay. Furthermore, rhizospheric and endophytic diazotrophic communities associated with rice roots seem sensitive to changes in rice rotation systems, which could influence the composition and diversity of these populations. To our knowledge, this is the first report on the impact of crop rotation on diazotrophic communities associated with rice roots under field conditions. Further studies should be conducted to deepen these findings and gain insights into the impact of crop rotations in these communities.

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Data availability The *nifH* gene raw data retrieved by 454-pyrosequencing in a previous work used in this study are available in the NCBI repository (https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA885822). Also, the *nifH* gene sequences retrieved by cloning in previous work were available (GenBank nucleotide accession numbers: KF872844–KF872993). All other data supporting the findings of this study are available within the paper and its Supplementary Information.

Declarations

Conflict of interest The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript. On behalf of all authors, the corresponding author states that there is no conflict of interest.

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