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Different Effects of Transgenic Maize and Nontransgenic Maize on Nitrogen-Transforming Archaea and Bacteria in Tropical Soils

Simone Raposo Cotta,[a,d] Armando Cavalcante Franco Dias,[b,d] Ivanildo Evódio Marriel,[c] Fernando Dini Andreote,[b] Lucy Seldin,[a] Jan Dirk van Elsas[d]

Universidade Federal do Rio de Janeiro, Instituto de Microbiologia Paulo de Góes, Rio de Janeiro, Brazil[a]; Departamento de Ciência do Solo, Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo, Piracicaba, São Paulo, Brazil[b]; Empresa Brasileira de Pesquisa Agropecuária, Centro Nacional de Pesquisas de Milho e Sorgo (EMBRAPA/CNPMS), Sete Lagoas, MG, Brazil[c]; Department of Microbial Ecology, Center for Ecological and Evolutionary Studies (CEES), University of Groningen (RUG), Groningen, The Netherlands[d]

The composition of the rhizosphere microbiome is a result of interactions between plant roots, soil, and environmental conditions. The impact of genetic variation in plant species on the composition of the root-associated microbiota remains poorly understood. This study assessed the abundances and structures of nitrogen-transforming (ammonia-oxidizing) archaea and bacteria as well as nitrogen-fixing bacteria driven by genetic modification of their maize host plants. The data show that significant changes in the abundances (revealed by quantitative PCR) of ammonia-oxidizing bacterial and archaeal communities occurred as a result of the maize host being genetically modified. In contrast, the structures of the total communities (determined by PCR-denaturing gradient gel electrophoresis) were mainly driven by factors such as soil type and season and not by plant genotype. Thus, the abundances of ammonia-oxidizing bacterial and archaeal communities but not structures of those communities were revealed to be responsive to changes in maize genotype, allowing the suggestion that community abundances should be explored as candidate bioindicators for monitoring the possible impacts of cultivation of genetically modified plants.

The relationship between plants and soil microbes is well established in the literature. Several microbial types or consortia are supposed or known to be able to exert strong effects on plant fitness (1–3). These relationships are often specific, suggesting that changes in plant genotype can have an impact on the association of specific microbial groups with plants, thus altering the composition of the rhizosphere microbiome (2). Based on this premise, several authors have suggested that genetic modifications of plants may modulate rhizosphere biodiversity and possibly impact the functioning of the rhizosphere (4–6). Therefore, microorganisms have been broadly used to determine the possible effects of genetically modified (GM) plants on the soil environment. Some authors have indeed reported modifications in the structure, composition, and abundance of particular (nontarget) microbial groups (6, 7). However, these effects were, in general, quite transient (6, 8, 9).

For the detection of relevant shifts in the soil microbiota, functional microbial groups that are of low redundancy are prime candidates, as (i) their roles may be critical for the maintenance of soil functioning and (ii) they may be more responsive to environmental factors than high-redundancy groups. This applies to both natural and managed agricultural soils. Several functional microbial communities have been shown to be sensitive to changes in environmental parameters (10), for example, those involved in the transformation of nutrients, like sulfur and/or nitrogen (11–13).

In terms of the nitrogen cycle, two processes may be forwarded as being strongly modulated by environmental parameters, i.e., nitrification (14) and nitrogen fixation (13). Nitrification occurs in two steps: the first and rate-limiting step is the oxidation of ammonia to hydroxylamine, which is further oxidized to nitrite. The second step, performed by nitrite-oxidizing bacteria, encompasses the oxidation of nitrite to nitrate (15, 16). The oxidation of ammonia is catalyzed by the enzyme ammonia monooxygenase, which is produced by a few (phylogenetically restricted) proteobacteria (ammonia-oxidizing bacteria [AOB]) and some archaea belonging to the phylum *Thaumarchaeota* (ammonia-oxidizing archaea [AOA]) (14, 17, 18). Nitrogen fixation is the biological reduction of atmospheric nitrogen into ammonia, a step that is essentially performed by bacteria that harbor the *nifH* gene, a key gene coding for the structural part of nitrogenase, nitrogenase reductase (19).

We hypothesized that the cultivation of GM maize instead of non-GM maize as a result of the modification would change the relationship between the plants and the targeted nitrogen-cycling organisms, causing modifications in the structure and abundance of ammonia-oxidizing and nitrogen-fixing communities in the rhizosphere. The objective of this work was thus to determine the possible differential assembly of the selected functional microbial groups in the rhizospheres of the GM maize and the parental (conventional) maize in a field experiment. We used culture-independent analyses to perform this survey, as these provide the best possible picture of the targeted communities, basing our analyses on proxies consisting of target genes for nitrifiers (AOA and AOB) and the target gene (*nifH*) for nitrogen fixers.

MATERIALS AND METHODS

Maize cultivars and experimental conditions. An experimental field located at EMBRAPA Milho e Sorgo in the city of Sete Lagoas in the state of

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Address correspondence to Lucy Seldin, lseldin@micro.ufrj.br.

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Minas Gerais, Brazil, was used. A detailed description of the field was provided by Cotta et al. (20). Replicate plots of two different soils, one typical for the Cerrado region in Brazil (a dark red acid dystrophic latosol with a clayey texture) and another commonly found in Várzea areas (a low-humus, eutrophic gley soil with a clayey texture and subneutral pH), were used. In these soils, two maize hybrids, both expressing a *Bacillus thuringiensis* insect toxin (Bt toxin), were used: (i) MON810 genotype 30F35Y (expressing the insecticidal Bt toxin Cry1Ab) and (ii) TC1507 genotype 30F35H (expressing the insecticidal Bt toxin Cry1F and the herbicide resistance phosphinothricin-*N*-acetyltransferase [*pat*] gene from *Streptomyces viridochromogenes*). The nearly isogenic parental line of each of the two GM maize cultivars was used as the control.

Plants were cultivated during the traditional period for maize cultivation in Brazil, known as safra (the second semester/harvest in August, during the rainy period of the year) and also during a nontraditional period for maize cultivation, known as safrinha (first semester/harvest in February, during the dry period of the year). Sampling expeditions were performed at three time points: during the transition from the vegetative to the reproductive stage of growth (30 days), at flowering (60 days), and during grain filling and maturity (90 days). At each time point, three sets of three plants of each cultivar were harvested. The roots of these plants were shaken manually to remove the loosely attached soil. The soil adhering to the roots of each set of three plants was then pooled, homogenized, and considered one sample of the rhizosphere soil. This yielded three replicates per treatment, and a total of 54 samples were analyzed (3 periods × 3 treatments × 2 periods of cultivation × 3 replications). The samples were kept at −20°C until DNA extraction and further analyses.

Determination of rhizosphere pH and ammonium and nitrate concentrations. The chemical characteristics of the rhizosphere samples were determined at EMBRAPA Milho e Sorgo, where the pH in water was determined as described by Silva et al. (21). Nitrate (NO_3^-) and ammonium (NH_4^+) contents were determined using a standard protocol described by Keeney and Nelson (22).

Extraction of DNA from rhizosphere samples. The total DNA of the microbial communities was extracted directly from the rhizosphere soil samples (0.5 g of each sample in triplicate) using a FastPrep Spin kit for soil DNA (Bio 101 Systems, USA). DNA preparations were visualized by electrophoresis in a 0.8% agarose gel in 1× TBE (Tris-borate-EDTA) buffer (23) to assess yield and integrity. The samples were then stored at −20°C prior to PCR amplifications.

Quantification of AOA, AOB, and diazotrophic communities by quantitative PCR (qPCR). The abundance of AOA, AOB, and diazotrophic organisms was estimated by using as proxies the *amoA* gene for archaea and bacteria and the *nifH* gene for diazotrophic organisms. Quantifications from each of the soil samples were carried out twice using an ABI Prism 7300 cycler (Applied Biosystems, Germany) with SYBR green I. The reactions were performed in 25-μl reaction mixtures containing 12.5 μl of the reaction mixture from a SensiMix SYBR kit (Bioline Life Sciences, Germany), 0.2 μM primers specific for each gene, 0.2 μg of bovine serum albumin (BSA), and 2 μl of target DNA (approximately 10 ng). Primers Crenamo23F (24) and CrenamoA616r48x (25) were used to quantify AOA, resulting in fragments of 624 bp. Thermal cycling consisted of 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 45 s, primer annealing at 50°C for 45 s, and elongation at 72°C for 45 s. Similarly, AOB quantification was performed using primers amoA-1F and amoA-2R as described by Nicol et al. (25). Amplifications were conducted according to the following cycle parameters: an initial denaturation step at 94°C for 10 min and 40 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. For the quantification of the *nifH* gene, primers FGPH19 and PolR were used, as described by Taketani et al. (26). The amplification conditions were an initial denaturation step at 94°C for 10 min, followed by 40 cycles of 1 min at 94°C, 27 s at 57°C, and 1 min at 72°C. The specificity of the amplification products was confirmed by melting curve analysis, and the sizes of the amplified fragments were checked in a 1.5% agarose gel stained with ethidium bromide. For quantification of the target genes, standard curves were constructed by performing amplification with a known number of copies of template DNA, serial dilutions of which were added to the reaction mixtures (10^7 to 10^2 copies to the reaction mixture for AOA, 10^8 to 10^1 copies to the reaction mixture for AOB, and 10^6 to 10^1 copies to the reaction mixture with *nifH*). The amplification efficiency was 100% for *amoA* from AOA, 109% for *amoA* from AOB, and 93.26% for *nifH*. The data from the amplification of rhizosphere DNA (the threshold cycle values) were interpolated to determine the number of copies of the target genes in the sample. Amplification of different amounts of target DNA (standard curve) also resulted in the determination of the amplification efficiency and logarithmic correlation (R^2) between the number of cycles and the amount of DNA.

PCR-denaturing gradient gel electrophoresis (DGGE) analysis of the AOA, AOB, and diazotrophic communities. The analysis of AOA was performed using 50-μl reaction mixtures containing 10 ng of genomic DNA and 0.2 mM primers Crenamo23F and CrenamoA616r48x, 2.0 mM $MgCl_2$, 250 nM deoxynucleoside triphosphates (dNTPs), and 5 U *Taq* polymerase in the buffer supplied by the manufacturer (Bioline Life Science, Germany). Amplification conditions were as follows: an initial denaturation step at 95°C for 5 min; 10 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C; 20 cycles of 30 s at 92°C, 30 s at 55°C, and 1 min 72°C; and a final extension of 10 min at 72°C. As a molecular marker for the AOB community, we used forward primers CTO-A/CTO-B/CTO-C and reverse primer CTO-R, specific for a region of the 16S rRNA gene of AOB of the betaproteobacterial subdivision (27). The reactions were carried out using Bioline *Taq* polymerase (Bioline Life Science, Germany) and the manufacturer's buffer. $MgCl_2$ was added to a final concentration of 2.0 mM, nucleotides were added at 250 nM, and BSA was added at 0.4 mg ml^{-1}. Amplification conditions were as follows: an initial denaturation step at 93°C for 1 min; 35 cycles of 30 s at 92°C, 1 min at 57°C, and 45 s at 68°C; and a final extension of 5 min at 68°C. The resulting amplicons were used as the templates in the second PCR with bacterial primers GC-341F and 518R (28). A 170-bp amplicon was generated in this reaction.

As a molecular marker for the diazotrophic community, we used the *nifH* gene, amplified in a nested PCR as described by Demba Diallo et al. (29). For the first reaction, the forward primer FPGH19 and the reverse primer PolR were used at a final concentration of 0.5 μM. The reaction was carried out using Roche *Taq* polymerase (Roche, Germany) and the manufacturer's buffer supplied with $MgCl_2$. The dNTPs were added to a final concentration of 200 nM and BSA was added at 0.4 mg ml^{-1} in a 25-μl volume. Amplification conditions were as follows: an initial denaturation step at 94°C for 5 min; 30 cycles of 1 min at 94°C, 1 min at 56°C, and 2 min at 72°C; and a final extension of 15 min at 72°C. The resulting amplicons were used as the template in the second PCR with forward primer PolF-GC clamped together with the reverse primer AQER. In this reaction, a 360-bp amplicon was generated with the same PCR mixture used in the first reaction. Amplification conditions were as follows: an initial denaturation step at 94°C for 5 min; 30 cycles of 1 min at 94°C, 1 min at 48°C, and 2 min at 72°C; and a final extension of 15 min at 72°C.

All amplification systems were validated through cloning and sequencing, as described by Dias et al. (14). Briefly, amplicons obtained from each primer set were cloned into the vector pGEM-T (Promega, Leiden, The Netherlands). Randomly selected clones were PCR amplified with the relevant primers and sequenced at the facilities of the University of São Paulo, Brazil (http://genfis40.esalq.usp.br/multi/) using the Sanger methodology. The resulting sequences were compared to database sequences using the BLASTN program (http://www.ncbi.nlm.nih.gov).

Subsequent DGGE analyses for AOA, AOB, and the *nifH* gene were performed using an Ingeny PhorU2 system (Ingeny, Goes, The Nether-

TABLE 1 Chemical characteristics of the rhizosphere samples used in this study[a]

Cropping season and maize sample	Cerrado soil			Várzea soil		
	pH in water	Ammonium concn (mg kg^{-1})	Nitrate concn (mg kg^{-1})	pH in water	Ammonium concn (mg kg^{-1})	Nitrate concn (mg kg^{-1})
Safra						
30 control	5.81 ± 0.18^{A}	21.88 ± 2.60^{C}	30.15 ± 3.20^{E}	6.97 ± 0.04^{B}	38.69 ± 3.00^{D}	15.75 ± 2.5
30 TC1507	5.57 ± 0.229^{A}	16.98 ± 0.86^{C}	45.96 ± 1.23^{E}	6.11 ± 0.35^{B}	19.17 ± 0.86^{D}	49.63 ± 2.5
30 MON810	5.57 ± 0.11^{A}	18.07 ± 0.55^{C}	50.55 ± 0.55^{E}	6.69 ± 0.0006^{B}	50.42 ± 3.80^{D}	44.16 ± 0.55
60 control	6.31 ± 0.28^{A}	18.69 ± 2.66^{C}	54.49 ± 2.49^{E}	7.15 ± 0.06^{B}	36.98 ± 1.79^{D}	32.21 ± 1.23
60 TC1507	5.86 ± 0.09^{A}	36.54 ± 2.53^{C}	53.35 ± 1.54^{E}	6.90 ± 0.06^{B}	35.23 ± 2.90^{D}	24.11 ± 1.79
60 MON810	6.29 ± 0.08^{A}	44.95 ± 7.73^{C}	39.35 ± 2.49^{E}	6.69 ± 0.03^{B}	32.47 ± 2.5^{D}	19.40 ± 2.68
90 control	6.49 ± 0.17^{A}	35.10 ± 2.22^{C}	31.99 ± 2.33^{E}	7.07 ± 0.03^{B}	29.93 ± 1.5^{D}	21.56 ± 2.18
90 TC1507	6.46 ± 0.01^{A}	50.11 ± 2.27^{C}	15.53 ± 2.24^{E}	6.84 ± 0.007^{B}	38.60 ± 2.5^{D}	14.79 ± 2.3
90 MON810	6.62 ± 0.11^{A}	53.35 ± 1.67^{C}	25.56 ± 2.56^{E}	7.17 ± 0.005^{B}	30.85 ± 2.66^{D}	16.89 ± 2.97
Safrinha						
30 control	6.67 ± 0.24^{A}	35.54 ± 0.86^{C}	16.89 ± 1.48^{E}	$6.79 \pm 5e\text{-}05^{B}$	40.31 ± 1.17^{D}	26.61 ± 0.86
30 TC1507	6.80 ± 0.0004^{A}	29.93 ± 2.84^{C}	21.97 ± 2.32^{E}	7.06 ± 0.03^{B}	45.17 ± 1.11^{D}	30.02 ± 1.998
30 MON810	6.48 ± 0.006^{A}	20.92 ± 2.08^{C}	29.46 ± 1.29^{E}	7.06 ± 0.01^{B}	36.63 ± 0.92^{D}	34.57 ± 0.86
60 control	6.67 ± 0.01^{A}	11.99 ± 2.22^{C}	25.25 ± 0.43^{E}	7.02 ± 0.001^{B}	30.72 ± 2.18^{D}	32.39 ± 1.60
60 TC1507	6.91 ± 0.06^{A}	11.77 ± 1.91^{C}	19.91 ± 3.03^{E}	6.93 ± 0.01^{B}	18.34 ± 1.54^{D}	22.71 ± 1.67
60 MON810	6.54 ± 0.001^{A}	9.86 ± 0.91^{C}	27.57 ± 0.97^{E}	6.93 ± 0.08^{B}	10.54 ± 1.29^{D}	28.59 ± 0.87
90 control	6.8 ± 0.14^{A}	11.43 ± 1.80^{C}	28.51 ± 0.88^{E}	7.22 ± 0.03^{B}	8.79 ± 1.17^{D}	27.51 ± 1.79
90 TC1507	7.17 ± 0.06^{A}	20.57 ± 2.24^{C}	29.06 ± 0.69^{E}	7.05 ± 0.08^{B}	9.74 ± 1.31^{D}	28.71 ± 1.97
90 MON810	7.05 ± 0.001^{A}	24.20 ± 0.80^{C}	25.78 ± 2.16^{E}	7.15 ± 0.02^{B}	15.32 ± 0.99^{D}	29.15 ± 2.3

[a] Values represent the averages of three repetitions and the standard deviations. Values followed by the same letter in columns indicate a statistically significant difference, determined by the Tukey test ($P < 0.05$). The values 30, 60, and 90 in the maize sample designations indicate 30, 60, and 90 days of cultivation, respectively.

lands) with denaturing gradients of 15 to 55%, 35 to 70%, and 45 to 65%, respectively (100% denaturant with 7 M urea and 40% formamide) and 6% polyacrylamide (14, 30). The DGGE runs were carried out at a temperature of 60°C, and the voltage was maintained at 100 V for 16 h. After electrophoresis, the gels were stained with SYBR Gold (final concentration, 0.5 mg $liter^{-1}$; Invitrogen, Breda, The Netherlands) and the gels were visualized on a photodocumented system under UV light.

Data analysis. The results of the quantitative PCR were analyzed by one-way analysis of variance, and average values were compared on the basis of the Tukey test at a significance level of 0.05 using the R package (RStudio, v0.97.312). Additionally, these results were correlated with environmental variables, such as soil characteristics, plant genotype, and temperature and the amount of rain (http://sigma.cptec.inpe.br/prec_sat/) during the period of the year that the crop was grown. Qualitative explanatory variables were transformed into dummy variables before analysis. Physicochemical variables were checked for normality, and all variables except for soil pH were log transformed. The respective effects of each explanatory variable or combinations thereof were determined by redundancy variation partitioning analysis with Canoco statistical software (Canoco, v4.5; Biometris, Wageningen, The Netherlands) (12, 31).

The band matrices derived from the PCR-DGGE patterns were analyzed to check the distribution of the data (linear or normal) through detrended correspondence analysis (DCA) (32, 33). To correlate the profile obtained with environmental variables (such as plant genotype, soil type, and period of cultivation), redundancy analysis (RDA) was used for data with a linear distribution (first gradient on DCA, <3.0), while data with a normal distribution (first gradient on DCA, <3.0) were analyzed by canonical correspondence analysis (CCA). In order to investigate the significance of the correlations between patterns and environmental variables, a Monte Carlo permutation test was performed on the basis of 999 random permutations; the null hypothesis was that species data were unrelated to environmental data (32, 34).

The influence of soil parameters on the abundance (from the qPCR data) of ammonia oxidizers and diazotrophic communities was determined by using Pearson's linear correlation coefficient implemented in SPSS (v16.0) software (SPSS, Inc., IL, USA).

Nucleotide sequence accession numbers. The sequences obtained in this study were deposited in the GenBank database with the following accession numbers: KJ866487 to KJ866491 for *amoA* from AOA, KJ866492 to KJ866496 for the 16S rRNA gene of AOB of the betaproteobacterial subdivision, and KJ866497 to KJ866501 for *nifH*.

RESULTS

Plant growth and rhizosphere parameters. All maize types tested (non-Bt toxin-expressing maize and Bt-toxin-expressing hybrids MON810 and TC1507) revealed similar growth rates during the safra and safrinha cropping seasons and in both soil types (Cerrado and Várzea). No signs of disease or nutrient scarcity were noted (data not shown).

Analysis of the rhizosphere soil showed differences in some chemical parameters between soil types and periods of plant cultivation (Table 1). Both soils revealed pH values that were largely subneutral, with the values for Cerrado soil ranging from 5.5 to 7.1 and those for the Várzea soil ranging from 6.1 to 7.1 ($P = 0.01$). Higher pH values were found in rhizosphere soil samples from the reproductive stage (growth to maturity, mainly during the safra period) (Table 1). The amounts of ammonium in the Cerrado soil ranged from 11.77 to 53.35 mg kg^{-1} (dry weight [dw]) soil, and those in the Várzea soil ranged from 9.7 to 50.42 mg kg^{-1} (dw) soil. In the Cerrado soil, we observed a distinct pattern along the cultivation period, with the safra period showing higher ammonia concentrations (16.98 to 53.35 mg kg^{-1} [dw] soil) and the safrinha period showing lower ones (9.86 to 35.54 mg kg^{-1} [dw] soil) ($P = 0.001$). In both soils, the ammonia levels during the safra period increased from the reproductive stage to the maturity

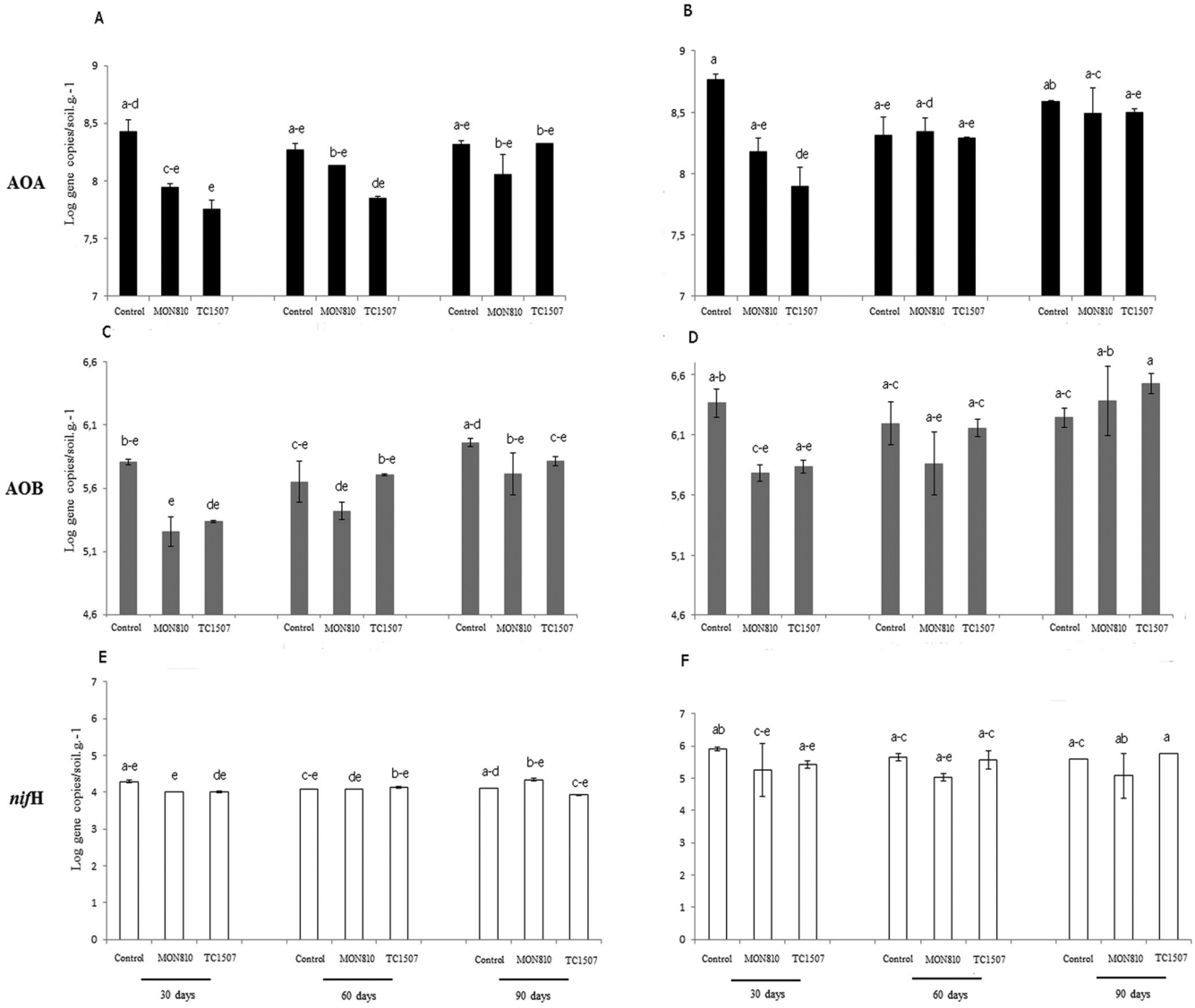

FIG 1 Abundance of ammonia oxidizers and diazotrophic communities in the rhizosphere of maize with distinct genotypes. (A, C, and E) Cerrado soil; (B, D, and F) Várzea soil.

stage, but during the safrinha period, the opposite trend was observed (Table 1). Similarly, nitrate levels showed higher values during the safra period (range, 15.5 to 54.0 mg kg^{-1} [dw] soil) than during the safrinha period (range, 16.8 to 29.6 mg kg^{-1} [dw] soil) ($P = 0.001$); again, decreased levels of nitrate from the reproductive stage to the maturity stage were found (Table 1).

Abundance of ammonia-oxidizing (AOA and AOB) and diazotrophic communities in the maize rhizosphere. Overall, the abundances of the quantified genes, *amoA* and *nifH*, were clearly affected by (i) maize genotype and (ii) the type of soil. In contrast, the gene copy levels did not show significant changes as a function of the period of maize cultivation, with very similar patterns being observed for the safra and safrinha periods (data not shown). The archaeal *amoA* gene (a proxy for AOA abundance) showed a higher abundance (between 7.5×10^8 and 8.8×10^9 gene copies g^{-1} soil) than the *amoA* gene of AOB (between 5.2×10^5 and 6.7×10^8 gene copies g^{-1} soil). Moreover, the *nifH* gene abundance was in the range of 3.9×10^4 to 6.0×10^6 gene copies g^{-1} soil (Fig. 1).

As suggested by the multivariate statistics, the main factors influencing the abundance of the AOA were plant genotype and soil type ($P = 0.001$) (Fig. 2). Variance partitioning indicated that 31.1% of the total variance in the abundance of the AOA communities could be explained by the physical and chemical characteristics of the soil and 14.9% could be explained by plant genotype (Fig. 2A). Considering the soil characteristics, pH and nitrate concentration were the main factors modulating the abundance of AOA; however, their contribution as drivers was modest. Variation in pH explained only 5% of the total variance, and pH had a positive correlation with the AOA abundance (+0.472). The nitrate concentration explained just 2% of the total variance and had a negative correlation with the abundance of AOA (−0.297). Maize genotype was also a key factor, as significantly reduced levels of AOA were found in the GM plant rhizosphere compared to

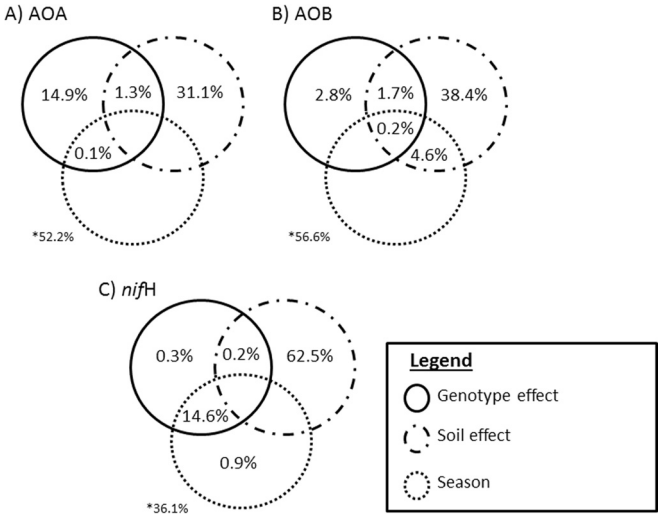

FIG 2 Interaction between environmental variables and abundance of ammonia oxidizers and diazotrophic communities in the rhizosphere of GM and non-GM maize plants. (A) AOA communities; (B) AOB communities; (C) diazotrophic communities. *, percentage not explained by variables used in this study.

the levels in the parental plant rhizosphere ($P = 0.001$) (Fig. 1 and 2).

Much like the results observed for the AOA, the AOB also showed significant differences in *amoA* gene copy numbers, which correlated with soil type and maize genotype ($P = 0.001$) (Fig. 1 and 2). Soil type had a more pronounced influence on the abundances of AOB than on those of the AOA, explaining 38.4% of the total variance. The effect of maize genotype was less pronounced, with only 2.8% of the total variance being explained by this variable (Fig. 2B). Concerning the physical and chemical characteristics of the soils, pH as well as the concentrations of nitrate and ammonia was correlated with the AOB abundances. The effect of pH was most pronounced (it explained 31% of the total variance), and pH had a positive correlation with bacterial *amoA* gene copy numbers (+0.560). On the other hand, the concentrations of nitrate (which explained 9% of the total variance) and ammonia (which explained 1% of the total variance) showed negative correlations (−0.362 and −0.112, respectively) with the AOB abundances in the maize rhizosphere. Although the maize genotype did not strongly affect the AOB abundances, an ephemeral effect of genotype could be observed (Fig. 1 and 2). The effect was also found in samples, mainly in Cerrado soil, collected 30 days after plant emergence, as the nontransgenic plants at that time revealed higher AOB abundances than the GM genotypes ($P = 0.001$) (Fig. 1C). This effect was also observed in the same soil sampled at 90 days. However, at this time point, the opposite was observed for the Várzea soil, where both transgenic plants showed higher AOB abundances than their nontransgenic counterparts (Fig. 1D).

Remarkably, the abundances of diazotrophs (measured by the use of *nifH* as a proxy) were strongly influenced by soil characteristics (which explained 62.5% of the total variance) (Fig. 1 and 2C) but not by plant genotype. Among the soil variables, pH and nitrate concentrations were the main drivers of *nifH* gene abundance. Variations in pH explained 21% of the variance, with pH having a positive correlation (+0.472) with *nifH* gene levels, while nitrate concentration accounted for only 3% of the variance, revealing a negative correlation (−0.205) with *nifH* gene abundance.

PCR-DGGE analyses of the AOA, AOB, and diazotrophic communities. The structures of the ammonia oxidizer (AOA and AOB) and nitrogen-fixing communities were assessed through specific PCR-DGGE analysis. The data showed that, overall, the three communities were clearly differentiated and structured by soil type. Moreover, plant developmental stage affected them, whereas plant genotype was not a driver. Detrended correspondence analysis (DCA), applied to analyze the data distribution, revealed values for the length of the first gradient of 6.37 (AOA), 2.89 (AOB), and 6.28 (*nifH*). Such values indicated that CCA for AOA and diazotrophic communities and RDA for AOB communities constituted the best mathematical models for multivariate analyses. The analysis thus performed showed that the ammonia oxidizer community structures grouped along soil type and season of sampling ($P < 0.05$) (Fig. 3A and B; Table 2). Specifically for the AOB community, a separation of the samples by pH was found (Fig. 3B). On the other hand, the diazotrophic communities showed a diffuse pattern of distribution and had only a slight responsiveness to pH values (Fig. 3C; Table 2).

Sequencing of the amplicons obtained with the three systems indicated (i) that all three amplification systems were specific for their target groups, i.e., AOA, AOB, and diazotrophic communities, and (ii) that the majority of the sequences analyzed were affiliated with as-yet-unclassified organisms. Specifically, all of the fragments generated with the ammonia oxidizer-specific primers were found to affiliate with uncultured AOA (AOA-specific system) and uncultured AOB (AOB-specific system). With respect to the diazotrophic communities, 90% of the sequences were indicated to originate from uncultured nitrogen-fixing bacteria.

DISCUSSION

The rhizosphere is considered to represent a so-called microbial activity hot spot and constitutes one of the most complex ecosystems on Earth because of the interplay of rhizosphere organisms (35). Together with the plant root, the local soil shapes the microbial diversity of the rhizosphere and also determines its functioning (2, 34, 36, 37). Host plants can thus direct the microbial composition in the immediate vicinity of their roots (38), a process which is possibly reigned by the host plant genotype. However, we lack information on the putative role of alterations in plant genotype that may shape the root-associated microbial communities. Genetic modification of plants constitutes such an alteration, which has a putative bearing on the organisms targeted by the modification, next to potential other (collateral) effects resulting from the modification. Thus, GM plant cultivation presents an interesting opportunity to evaluate the fundamental science of the rhizosphere, as well as represents a highly practical test case, as it constitutes a clear effort toward the development of sustainable agriculture (enabling the cultivation of plants with the reduced use of pesticides and fertilizers) but may also have adverse consequences.

In this study, performed in two soils and during two cultivation periods, we addressed several parameters and their effects on selected nitrogen-transforming communities in the maize rhizosphere. Our results revealed that the host plant had a clear effect on the abundance of the ammonia-oxidizing communities during

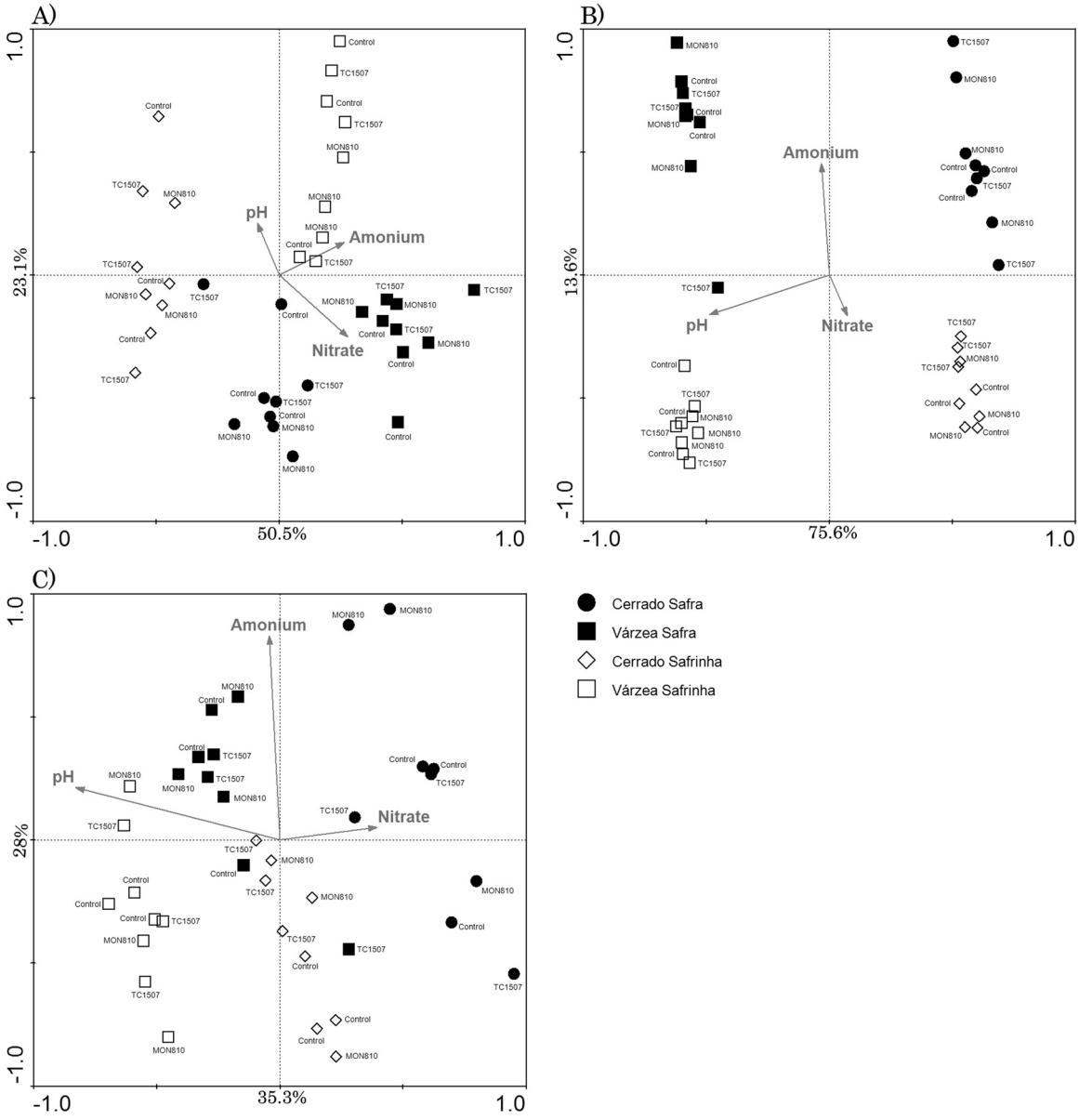

FIG 3 Ordination analyses of the drivers for the structure of ammonia oxidizers and diazotrophic communities in the rhizosphere of maize plants of distinct genotypes. (A) AOA communities; (B) AOB communities; (C) diazotrophic communities. The number on each axis indicates the percentage of the total variation explained.

the transition from the vegetative to the reproductive stages of plant growth (30 days) and grain filling and maturity (90 days). At these time points, the two GM plants apparently incited significantly lowered abundances of AOA and AOB than the corresponding parental lines. In other studies, the responses of such communities have been attributed to shifts in soil characteristics, such as pH, temperature, and ammonia concentration (18, 24, 25). We cannot easily explain this effect seen by us, but we surmise that it may be related to altered plant physiology due to the genetic modification, resulting in quite strongly altered local conditions in the rhizosphere (e.g., alterations in ammonia availability right at the root surface, which is hard to measure experimentally) with which the plant-associated AOA and AOB are confronted. In previous work, differential structuring of ammonia-oxidizing communities was also shown by Song et al. (39), who attributed the differences to something that they described as a general distinction with respect to root exudation and/or residue composition. Icoz et al. (40) had similar results when analyzing AOB, finding lower abundances in soils cultivated with their transgenic maize (event Bt11) than in soils cultivated with nearly isogenic nontransgenic maize plants. In related work, the authors found evidence for the differential degradation of compounds derived from *B. thuringiensis*-treated maize and those derived from nontransgenic maize (41, 42). As matter derived from *B. thuringiensis*-treated maize showed lower degradability, the *B. thuringiensis*-treated maize supposedly releases smaller amounts of ammonium into the rhizosphere, thus limiting the abundance of ammonia-oxidizing organisms. Indeed, in our study, the ammonium levels

TABLE 2 Statistical analyses of PCR-DGGE data obtained by multivariate analyses, where matrices made of band patterns were integrated with environmental data

Microbial community (type of analysis)	Environmental variable	Lambda A^a	*P* value	*F*
AOA (CCA)	Type of soil	0.45	0.002	5.15
	Period of cultivation	0.27	0.002	3.20
	pH	0.13	0.084	1.66
	Ammonium	0.13	0.076	1.64
	Nitrate	0.11	0.122	1.36
AOB (RDA)	Type of soil	0.44	0.002	27.10
	Period of cultivation	0.08	0.002	4.97
	pH	0.03	0.004	2.63
	Ammonium	0.02	0.134	1.39
	Nitrate	0.002	0.226	1.30
Diazotrophic community (CCA)	Type of soil	0.34	0.434	1.05
	Period of cultivation	0.41	0.188	1.24
	pH	0.58	0.018	1.71
	Ammonium	0.48	0.058	1.45
	Nitrate	0.27	0.698	0.80

[a] Lambda *A*, additional variance.

in the rhizosphere of the GM plants was reduced compared to those in the parental line (mainly at 30 days), corroborating this hypothesis. The hypothesis that variables like the ammonium level (next to pH) are major drivers of ammonia oxidizer assemblages in soils (38, 43) is thus extended to the rhizosphere.

However, one might also forward the hypothesis that altered root exudate patterns are the main modulators of the target organism abundances. Even considering that AOA are supposed to be chemolithotrophs, they may be selectable due to the release of specific C_1 compounds from the roots (44). Moreover, indirect selection can also take place, as root exudates, e.g., exudates containing particular organic acids, can modulate the local environmental conditions, such as pH (39).

The observations on the abundances of AOA and AOB were not corroborated by those on the structures of such communities. In other words, where abundances changed, community structures remained rather similar. Thus, the abundance responses that we observed should be regarded as overall responses rather than the responses of particular types within the target communities. However, our results indicate that the groups composing these communities are mostly determined by factors other than plant genotype. The PCR-DGGE patterns were clearly driven by soil type as well as, for each soil type, by cultivation period (sampling time). In recent work, Dias et al. (9, 14) described just slight shifts in the composition of AOA and AOB communities between the rhizospheres of nontransgenic and GM (modified to have an altered starch allocation) potato cultivars. Thus, we may surmise that plant genotype might not be a strong driver of AOA/AOB community structures compared to the strength of other drivers, such as soil type and season/climate (15). Unfortunately, we were unable to pinpoint defined taxonomic groups underlying the AOA/AOB communities in each of the rhizospheres. Rather, we found a prevalence of organisms related to unclassified groups of AOA and AOB, highlighting these to be prime targets for future studies.

The assessment of diazotrophs, which here was based on estimates of the abundances and community structures determined using the *nifH* gene, indicated that drivers other than plant genotype were the major effectors of these communities. The abundances of the *nifH* gene in the low-pH Cerrado soil were significantly lower than those in the more neutral Várzea soil, corroborating the finding of lower rates of nitrogen fixation in soils with lower pH (30, 45). The community structure data indicated that soil type and associated parameters, rather than plant genotype, were major drivers of the diazotrophic communities, much like the result observed for AOA and AOB. Considering that plant roots and rhizospheres are expected to constitute potential drivers of nitrogen fixers (a clear example is given by the rhizobial types, including *Bradyrhizobium*, which are strongly favored by rhizospheres), one tends to conclude that the potential differences between the GM types and the parental type in our study were not effective in inducing such changes. Given the fact that the rhizosphere community structures were not driven by plant genotype, we did not apply extensive sequencing to characterize these communities.

On the basis of our data as well as previous data, we suggest that our target groups, in particular, AOA, can be used as bioindicators to further explore the disturbances or potential impacts caused by GM plants. These organisms appear to show a ready differential response to the effects of plant roots, which goes against the finding that soil communities are often resilient or resistant to disturbance due to their substantial functional redundancy. Previous studies already indicated the use of, in particular, ammonia oxidizers as bioindicators (11, 13, 15). Our current study extends this notion to GM maize plants grown in tropical soils by affirming that two different GM maize plants incited changes in AOA and AOB abundances in their rhizospheres, whereas the structures of these communities remained unaffected. However, it is obviously unclear what the long-term impacts of such alterations in potential ammonia oxidation function are, and insight into the ecology of soil function suggests that the effect is very likely quite ephemeral.

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