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Effect of Herbicide Combinations on Bt-Maize Rhizobacterial Diversity

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Reports of herbicide resistance events are proliferating worldwide, leading to new cultivation strategies using combinations of pre-emergence and post-emergence herbicides. We analyzed the impact during a one-year cultivation cycle of several herbicide combinations on the rhizobacterial community of glyphosate-tolerant Bt-maize and compared them to those of the untreated or glyphosate-treated soils. Samples were analyzed using pyrosequencing of the V6 hypervariable region of the 16S rRNA gene. The sequences obtained were subjected to taxonomic, taxonomy-independent, and phylogeny-based diversity studies, followed by a statistical analysis using principal components analysis and hierarchical clustering with jackknife statistical validation. The resilience of the microbial communities was analyzed by comparing their relative composition at the end of the cultivation cycle. The bacterial communites from soil subjected to a combined treatment with mesotrione plus s-metolachlor followed by glyphosate were not statistically different from those treated with glyphosate or the untreated ones. The use of acetochlor plus terbuthylazine followed by glyphosate, and the use of aclonifen plus isoxaflutole followed by mesotrione clearly affected the resilience of their corresponding bacterial communities. The treatment with pethoxamid followed by glyphosate resulted in an intermediate effect. The use of glyphosate alone seems to be the less aggressive one for bacterial communities. Should a combined treatment be needed, the combination of mesotrione and s-metolachlor shows the next best final resilience. Our results show the relevance of comparative rhizobacterial community studies when novel combined herbicide treatments are deemed necessary to control weed growth.

Keywords: 16S rRNA, herbicide, maize, metagenomics, rhizobacteria, *Zea mays*

Introduction

Numerous studies have shown that weeds are able to significantly reduce maize yield (between 35% and 60%), particularly during the so-called critical period of weed control [22, 38], thus leading to the widespread use of herbicides to control the growth of competing, undesirable weeds. Herbicide usage has been a common agricultural practice since the middle of the 20th Century, and over the years has led to the emergence of multiple resistance events in weeds (218 species tolerant to 148 different herbicides as of October 2013 are reported in http://www.weedscience.org). To address this problem, seed companies have resorted to producing new recombinant crops tolerant to broader-range non-selective herbicides such as glyphosate. However, owing to the continued use of these products, new weed tolerance events are increasingly taking place as well [37], and the development of new strategies for weed control is becoming more relevant, such as combinations of pre-emergence and post-emergence herbicides in the same seeding-harvest cycle, often in conjunction with the cultivation of genetically modified plants.

Herbicides are strong chemical products that not only act on their target weeds but may also display significant toxicity to other organisms [14, 42]; among these, the bacteria present in the rhizosphere, which potentially affect plant growth, herbicide degradation capacity of the soil, and the ability of soils to improve crop yield [13, 25].

Plant growth-promoting bacteria (PGPB) and rhizobacteria (PGPR) community diversity and evenness have been reported to increase yield by stimulating plant growth, facilitating plant adaptation to adverse or stressing environments, and by reducing susceptibility to pathogen infection by means of a variety of mechanisms. PGPR are plant and soil specific and have been used successfully to facilitate plant seeding, rooting, growth, and yield under aggressive conditions [13].

The structure of the specific maize rhizobacterial community displays a stronger dependence on soil properties than on cultivar variety. The maize rhizosphere has been shown to be enriched in Proteobacteria, particularly Burkholderiales, which are known to respond to labile carbon sources and are considered r-selected, fast growing, and opportunistic, in contrast to bulk soil, which is enriched in k-selected, slower growing microbia [40]. Actinobacteria play a major role in soil owing to their ability to decompose organic materials and secrete growth-promoting substances, antibiotics, enzymes, and enzyme inhibitors, in addition to many other compounds [27]. Acidobacteria and Verrucomicrobia, although difficult to cultivate and less well known, are ubiquitous, particularly in soils, and are likely to play a relevant role in terrestrial ecosystem processes [33].

The potential effects of the specific herbicides and their dosage, and of soil properties and maize variants on the rhizobacterial communities have been the subject of several short- and long-term studies since their introduction (*e.g.*, [10, 17, 19]). Interestingly, recent studies using Next Generation Sequencing (NGS) and metagenomics have confirmed the frail impact of the Cry1Ab protein [8] or soil properties [7] on the effects of glyphosate, which in turn shows little impact both in cotton [6] and maize [4, 5] in contrast to the tank mix (combination in a single formulation) of acetochlor plus terbuthylazine [3]. Given its weaker impact, glyphosate is the obvious choice for use in combined treatments.

Existing studies have produced significant insights into the relative impact of each agent and have shown that tank mix use of herbicide combinations may display significant antagonistic, synergistic, or additive effects [43], and have suggested that time-separated administration might mitigate their combined effects. However, until now there is very little knowledge as to the effects of the combination of pre- and post-emergence applications. Given this scarce knowledge, we have analyzed the potential impact of new strategies on rhizobacteria combining the pre-emergence application of various herbicides with the post-emergence treatment with glyphosate.

Materials and Methods

Herbicides Tested

We have considered the use of various time-separated commercial herbicide preparations in combination with glyphosate (hence requiring a glyphosate-tolerant maize variant).

Two control demonstration fields were seeded: one field contained solely Bt-maize with no treatment at all, and a second field was treated only in post-emergence with 360 g/l of glyphosate as isopropylamine salt (Roundup Ready, 72 kg/ha).

Three experimental demonstration fields were treated, each one in pre-emergence, with a commercial preparation of either pethoxamid 60% (Successor, 2 l/ha), a combination of mesotrione 4% and s-metolachlor 40% (Camix, 3 l/ha), or a combination of acetochlor 41% plus terbuthylazine 19.5% (Harness GTZ, 3 l/ha), and were subsequently treated in post-emergence with glyphosate, as described.

An additional demonstration field was cultivated using a combined treatment suitable for conventional, non-glyphosate-tolerant maize consisting of a combination of aclonifen 50% plus isoxaflutole 7.5% (Lagon, 1 l/ha) applied pre-emergence, followed by mesotrione 10% (Callisto, 1 l/ha) applied post-emergence. All doses used correspond to current standard practices.

Texture and Chemical Properties of the Soil

Maize samples were grown in experimental demonstration fields located in Ejea de los Caballeros (42º 2′ 16′′ N, 1º 13′ 47′′ W, Zaragoza, Spain). The surface of each experimental demonstration field measured 40 m^2, and all of the demonstration fields were annexed to each other and separated by a 4-m-wide path.

The agricultural soil texture and composition were uniform across all of the demonstration fields and composed of clay loam consisting of 43% sand, 29% clay, and 28% silt, with 3.27% oxidable organic matter (Wakey-Black), a pH of 8.17, a cationic exchange capacity of 15.20 meq/100 g, and an electrical conductivity of 242 μS/cm at 20ºC, as determined by the independent contractor Labs & Technological Services AGQ, S. L.

Since all soils share the same properties, we used typical values of DT_{50} (a measure of contaminant degradation time) and K_{FOC} (based on the Freundlich isotherm and normalized for organic carbon values) to discuss the differences in herbicide leaching and soil sorption properties (Table 1), as recommended by FOCUS (http://focus.jrc.ec.europa.eu/gw/docs/).

Plant Material and Sampling

Previous studies [4, 5, 40] have confirmed that regional conditions exert the largest impact on bacterial biodiversity, and that, within each location, differences due to the herbicide treatment are the most relevant, indicating that comparisons should be based on relative differences within each location [7]. It has also been shown that maize production of the Cry1Ab protein exerted a minor or undetectable effect [8]. To reduce any possible confounding variables in the study, all experiments and samplings

Table 1. Herbicide leaching properties[a].

Name	Family	Solubility[b] (H_2O, 20ºC)	Soil adsorption and mobility (Freundlich) K_{FOC}	Typical 50% dissipation time[c] DT_{50}
Glyphosate	Phosphonoglycine	10,500	28,700	12
Pethoxamid	Chloroacetamide	400	154	6
Mesotrione	Triketone	160	53	32
S-Metolachlor	Chloroacetamide	530	226.1	15
Acetochlor	Chloroacetamide	282	285	14
Terbuthylazine	Triazine	6.6	231	75.1
Aclonifen	Diphenyl ether	1.4	7,126	117
Isoxaflutole	Isoxazole	6.2	112	2

[a]Values for relevant herbicide leaching properties were obtained from the Pesticide Properties Database website (http://sitem.herts.ac.uk/aeru/projects/ppdb/).

[b]Solubility in water at 20ºC is expressed as mg/l.

[c]Typical 50% dissipation time (DT_{50}) values for each herbicide, expressed in days.

were conducted simultaneously, using the same maize variant, in demonstration fields with the same soil, hence comprising the same texture, NPK composition, and properties, and subject to the same environmental and climatic factors as well as the same agricultural practices.

Equivalent numbers of plants were used in each of the experimental fields: Glyphosate-tolerant Bt-maize, event NK603, MON810, variety DKC6451YG (Monsanto Agricultura, Spain), expressing the Cry1Ab Bt toxin that confers resistance to the European corn borer was grown using current agricultural practices throughout the cultivation period.

In order to deal with local soil micro-heterogeneity, and given the small size of the demonstration fields, these were divided into nine randomized subplots, and three subsamples from each subplot were taken at each sampling time. Equal amounts of soil (10 g) from the 27 extractions from each maize field at each sampling time were pooled for the subsequent analysis of each soil to obtain an average figure of the rhizobacterial community in each sample. No appreciable differences in plant weight, length, above-ground weight, number of leaves, root system, or yield were detected among the plants grown in the different fields at any given sampling time. Hence, the differences in the results are expected to be due to the differences in the herbicide treatments on the rhizosphere bacterial community.

We use the following terminology for fields according to the corresponding treatment applied: C (control field), G (glyphosate only), PG (pethoxamid and glyphosate), MSG (mesotrione plus s-metolachlor and glyphosate), ATG (acetochlor plus terbuthylazine and glyphosate), and AIM (aclonifen plus isoxaflutole and mesotrione). Samples are termed according to the field, followed by a number (1 or 2 depending on the sampling time), hence C1 stands for "control field, first sampling time."

DNA Extraction, PCR Amplification, and Pyrosequencing

During the course of 2012, samples were harvested from each field at two different stages: soon after emergence when the plants were shorter than 10 cm and had 2–3 leaves (first sampling time), and just before crop harvesting, approximately six months after seeding (final sampling time). The root system and adhered soil measuring approximately 2 mm or less in diameter were shaken gently to separate bulk soil. Soil adhered to these roots was carefully extracted to separate the rhizosphere from the plant roots.

The rhizospheres from each field at each collection time were subjected to three independent DNA extractions using PowerMax Soil DNA kits (MO Bio Laboratories Inc., Carlsbad, USA) following the protocol provided by the manufacturer. DNA obtained from each of these three independent extractions was used as template for PCR amplification of the V6 region of the 16S rRNA gene. The oligonucleotide design included 454 Life Science's Titanium A (5′- CGTATCGCCTCCCTCGCGCCATCAG-3′) or B (5′-CTATGCGCCTTGCCAGCCCGCTCAG-3′) pyrosequencing adaptors fused to the 5′ end of the template-specific primers 967F (CAACGCGAAGAACCTTACC) or 1046R (CGACAGCCATGC ANCACCT), and a sample-specific MID (multiplex identifier) immediately preceding the V6-specific primer (Table 2). Soil DNA was used as template for PCR amplification with incubation at 95ºC for 5 min, followed by 30 cycles of incubation at 95ºC (30 sec), 63ºC (45 sec), and 72ºC (1 min), with a final extension cycle of 5 min at 72ºC [3]. The resulting DNAs obtained from the three independent PCRs were pooled and cleaned using an Illustra GFX PCR DNA Amplification kit (GE Healthcare), checked with Bioanalyzer 2100 (Agilent Technologies), quantified with Quant-IT-picogreen (Invitrogen), and used to make the single strands on beads, as required for 454 Titanium pyrosequencing [35]. Pyrosequencing was conducted using a 454 Life Sciences Titanium pyrosequencer (Life Sequencing S. L. and the Research Centre for Public Health, CSISP, Valencia, Spain). Bar coding allowed us to analyze two samples per single 454 available lane (Table 2).

Table 2. Samples and bar codes used.

Sample	Field[a]	Time[b]	Treatment: pre-emergence[c]	Treatment: post-emergence[d]	Multiplex identification tags[e]	Reads[f]
1	C	1	None (control)	None (control)	ACGCTCGACA	11,791
2	G	1	None	Glyphosate	AGACGCACTC	31,894
3	PG	1	Pethoxamid	Glyphosate	AGCACTGTAG	28,366
4	MSG	1	Mesotrione + s-metolachlor	Glyphosate	ATCAGACACG	10,721
5	ATG	1	Acetochlor + terbuthylazine	Glyphosate	ATATCGCGAG	7,660
6	AIM	1	Aclonifen + isoxaflutole	Mesotrione	CGTGTCTCTA	4,709
7	C	2	None (control)	None (control)	CTCGCGTGTC	91,627
8	G	2	None	Glyphosate	TAGTATCAGC	50,353
9	PG	2	Pethoxamid	Glyphosate	TCTCTATGCG	37,922
10	MSG	2	Mesotrione + s-metolachlor	Glyphosate	TGATACGTCT	37,475
11	ATG	2	Acetochlor + terbuthylazine	Glyphosate	TACTGAGCTA	44,138
12	AIM	2	Aclonifen + isoxaflutole	Mesotrione	CATAGTAGTG	42,216

[a]Experimental fields have been labeled according to treatment (C: control; G: glyphosate; PG: pethoxamid and glyphosate; MSG: mesotrione plus s-metolachlor and glyphosate; ATG: acetochlor plus terbuthylazine and glyphosate; AIM: aclonifen plus isoxaflutole and mesotrione).

[b]The "Time" column indicates sampling time: 1 (soon after emergence) or 2 (late before harvest).

[c]Pre-emergence and [d]post-emergence herbicide treatments applied to each field.

[e]Multiplex identification tags are the oligonucleotides used to separate the sequences obtained from each sample.

[f]Reads indicate the number of sequences recovered from each sample.

Metagenomic Sequence Analysis

The sequence data were quality-filtered at various levels: first, allowing 3′ trimming (trim back valley filter), followed by a second step using QIIME [11] with a quality score window of 50, allowing for no ambiguity and truncating sequences at the reverse primers. After splitting the sequences according to their bar code, chimeras were detected with Otupipe's UCHIME [18] using *de novo* and Gold database-based reference filtering and removed. The remaining sequences were then compared using BLAST [1] against the SILVA database release 92 [41] for taxonomical classification. BLAST output files were used to generate the corresponding taxonomic trees by the Lowest Common Ancestor (LCA) method using MEGAN [28] and NCBI taxonomy. Observed differences in the relative proportions of taxa among samples were statistically validated, normalizing the data and using the Directed Homogeneity test with both the Bonferroni and Holm-Bonferroni corrections for multiple samples using MEGAN. Only differences among major taxa with p-values < 0.05 were considered for discussion.

An additional taxonomy-based filtering step was applied next: reads identified as contaminants by taxonomy (Eukaryota and Archaea), typically less than 0.5%, were removed from the analysis. From the remaining reads, sequences that could not be assigned to specific bacterial taxa were separated and clustered using Otupipe [18] to identify and remove singletons, which are most likely considered errors [44]. The remaining unassigned reads were pooled back with the other bacterial sequences for subsequent analysis.

To perform the alpha diversity analysis, QIIME was used to identify observed OTUs (Operational Taxonomic Units) and to compute the Chao1 estimates and the Shannon-Wiener Index as well as their respective rarefaction curves at 97% similarity (conventionally considered equivalent to species-level). Given the impossibility of comparing values beyond the minimal read depth, an amount of 4,000 reads from the first sampling time and of 30,000 reads from the final sampling time were selected as a balance, and a bootstrap stratified by treatment and sampling time using 50 randomly selected subsets from each sample was used to calculate the average and 95% confidence interval of the mean for each value.

For the beta-diversity analyses (comparison among populations), we compared the filtered sequences against Greengenes [15] using BLAST to obtain a mapping against the Greengenes reference phylogenetic tree to be used in the calculation of continuous weighted UniFrac metrics [24]. The matrix of UniFrac values obtained was used to carry out a Principal Components Analysis (PCA). The statistical significance of the relationships observed between groups was assessed by hierarchical clustering and determination of statistical support for each clustering step (branches in the sample grouping hierarchy) using jack-knifing with 1,000 permutations, keeping 2,100 sequences and using normalized abundance weights to account for uneven sample size.

The sequences obtained have been deposited in the EBI SRA archive (study accession number PRJEB4333).

Results

Taxonomical Analysis

The taxonomic breakdown of the more relevant phyla for each sample is shown in Fig. 1 and Table S1. On average, 32% of the reads could not be assigned to specific taxa below the Bacteria level in each sample. The assigned reads were used to compare the relative amounts of the different taxa among the samples. Only changes with highly significant *p*-values in the corrected Directed Homogeneity

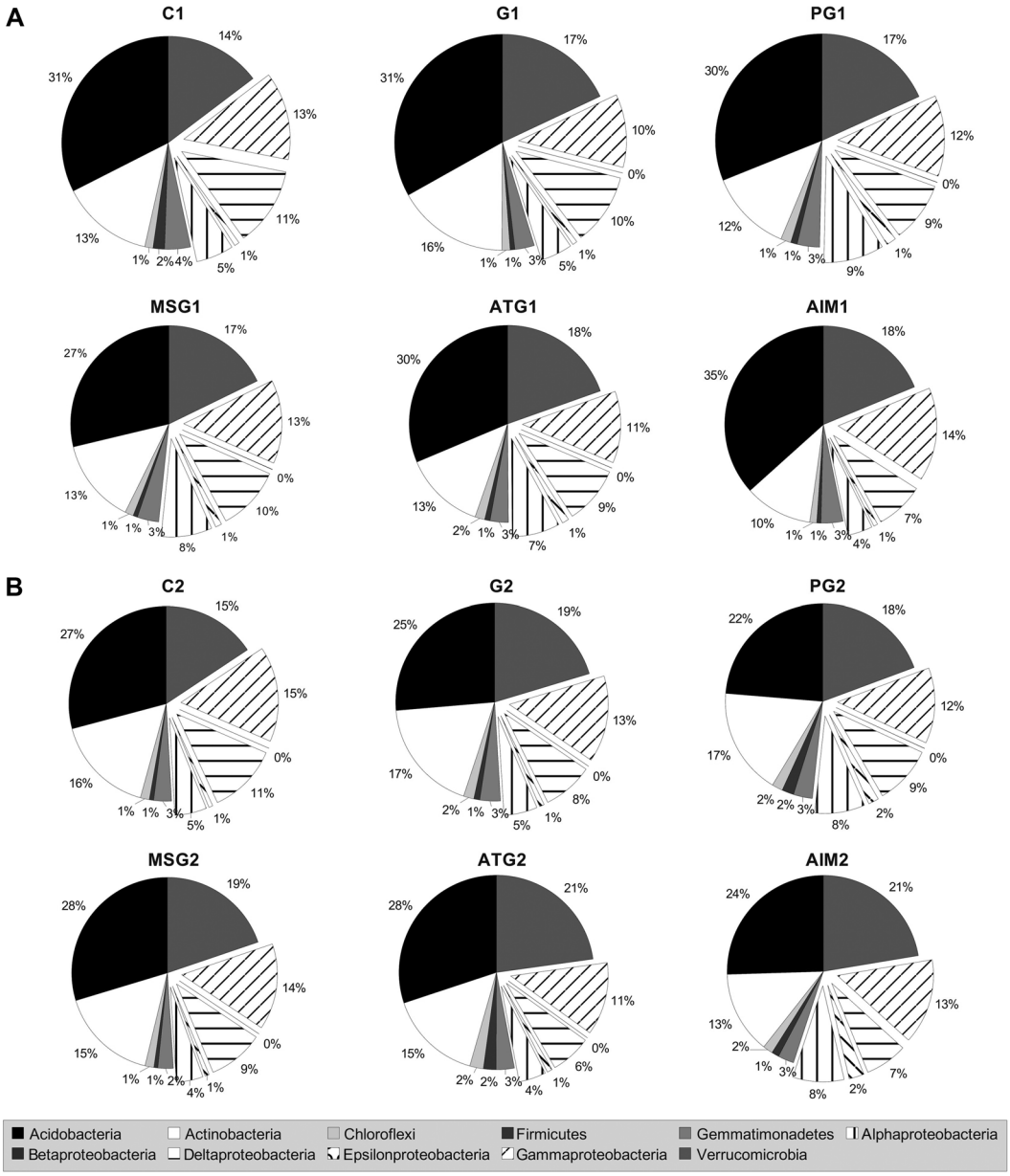

Fig. 1. Taxonomical breakdown resulting from pyrosequencing region V6 of the 16S rRNA of the rhizobacterial communities of glyphosate-tolerant Bt-maize subject to various herbicide combinations.

Rhizobacterial community samples were collected at two different sampling times from soils subject to different herbicide treatments (C: control; G: glyphosate; PG: pethoxamid and glyphosate; MSG: mesotrione plus s-metolachlor and glyphosate; ATG: acetochlor plus terbuthylazine and glyphosate; AIM: aclonifen plus isoxaflutole and mesotrione). The taxonomical breakdown at the phylum level of each sample rhizobacterial community at the initial (**A**) and final (**B**) sampling times is displayed as a pie chart. The most relevant phyla (presence above 1%) are charted with percentages rounded to the closest units displayed next to them.

test were considered (*p*-values have been omitted for simplicity).

The changes between C1 and C2 reflect the influence of plant growth on the relative composition of the rhizobacterial community, showing that most major phyla experienced an increase, with the exception of Acidobacteria whose relative presence decreased, thus providing the baseline for the rhizosphere changes induced by plant growth.

Treatment with glyphosate alone (G) provided a second reference to identify the additional effects of the combination treatments. Sample G1 showed a reduction in Proteobacteria, compensated by minor increases in Verrucomicrobia and Actinobacteria with respect to the untreated soil. In G2, Acidobacteria experienced a major reduction, compensated by increases in Verrucomicrobia, Actinobacteria, and Proteobacteria that brought the community structure closer to that of C2.

In the ATG field, the herbicide combination resulted initially (ATG1) in a decrease in Proteobacteria and Acidobacteria, counterbalanced by an increase in Verrucomicrobia and other changes in minor phyla with respect to the untreated soil. After plant growth (ATG2), Proteobacteria and Acidobacteria continued decreasing, while the presence of Verrucomicrobia and Actinobacteria increased. A comparison between ATG2 and C2 indicated that Proteobacteria had experienced a large reduction, opening a gap that was compensated by increases in other groups, mainly Verrucomicrobia. Compared with G2, the addition of acetochlor and terbuthylazine resulted in a 2-fold reduction of Proteobacteria, accompanied by a reduction of Actinobacteria, confirming that the effects were worse than the treatment with glyphosate alone.

The PG soil initially (PG1) showed a reduction of Actinobacteria, compensated by an increase in Verrucomicrobia and an alteration in the formula of Proteobacteria, with a decrease in Deltaproteobacteria matched by an increase in Alphaproteobacteria. After plant growth (PG2), Acidobacteria decreased significantly and were counterbalanced by the growth of Actinobacteria, Firmicutes, and Verrucomicrobia. Compared with C2, Acidobacteria also showed a significant reduction, confirming them as the most affected group, while Verrucomicrobia and Actinobacteria increased and Proteobacteria were less affected. When compared with G2, Verrucomicrobia and Acidobacteria decreased and Proteobacteria increased. In summary, the combination of pethoxamid and glyphosate mainly affected the Acidobacteria population.

The MSG field experienced changes between MSG1 and MSG2, with a decrease in Proteobacteria, compensated by various increases in other major phyla. The comparison between MSG1 and C1 revealed that the early effects led to a decrease in Acidobacteria and an increase in Verrucomicrobia and Proteobacteria, suggesting that this combined action mainly affects Acidobacteria. A comparison between MSG2 and C2 showed a decrease in Proteobacteria, with Deltaproteobacteria and Gammaproteobacteria being most affected, counterbalanced mainly by increases in Verrucomicrobia. Compared with the G field, the main difference between MSG1 and G1 consisted of a greater reduction in Acidobacteria, whereas at the final sampling time Acidobacteria showed a heightened presence.

The use of a conventional treatment for non-glyphosate-tolerant maize (field AIM) shows that between the first sampling time (AIM1) and after plant growth (AIM2) there was a very large decrease in Acidobacteria. A comparison with C1 revealed decreases in Actinobacteria, Proteobacteria, and other minor phyla, counterbalanced by an increase in Verrucomicrobia and Acidobacteria. At the final time (AIM2 *vs*. C2), Acidobacteria continued to experience a moderation accompanied by reductions in Actinobacteria, with Verrucomicrobia filling most of the gap.

Phylogenetic Analyses

The results of the PCA are indicated in Fig. 2. As expected, since the environmental properties were the same for all fields, the first principal component (PC1), explaining a 32.95% variation, is clearly associated with sampling time, with samples grouped separately from those collected at the final time; the second component (PC2), explaining a 19.08% variation, separates the samples in two parts: at the first sampling time one part groups together soils PG1 and ATG1, and the other part groups all the remaining soils, while at the final sampling time, the soils are spread across the second principal component, with C2 at one extreme and the remaining soils progressively separating from it in the following order of increasing distance: MSG2, G2, ATG2, PG2, and AIM2.

Hierarchical Clustering Analysis

The hierarchical clustering tree obtained using the Unifrac metric with jackknife validation is shown in Fig. 3, with nodes labeled according to their statistical support. Jackknifing values represent a combination of support for the branching event and the maximum-likelihood distance. The statistical validation further supports the above observations by separating samples according to collection time (statistical support of 100%) in agreement with PC1, thereby helping to classify the impact of the treatments

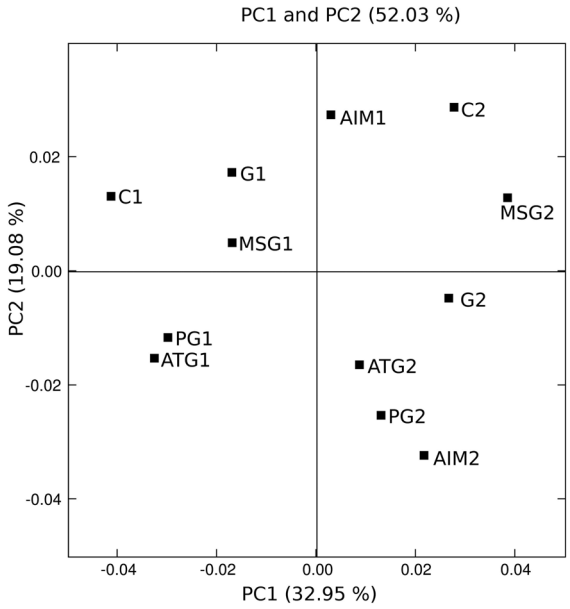

Fig. 2. UniFrac Principal Components Analysis of rhizobacterial communities from glyphosate-tolerant Bt-maize subject to different herbicide combinations.

Abbreviations are used to identify the treatments applied to each sample (C: control; G: glyphosate; PG: pethoxamid and glyphosate; MSG: mesotrione plus s-metolachlor and glyphosate; ATG: acetochlor plus terbuthylazine and glyphosate; AIM: aclonifen plus isoxaflutole and mesotrione), followed by a number identifying the sampling time (1: soon after emergence; 2: late before harvest). Samples with similar bacterial communities are localized in similar positions in the diagram. Dispersion across the first component, P1 (explaining 32.95% of the variation), clearly groups samples by collection time, while dispersion across the second component, P2 (19.08% of the variation), shows how samples group at each sampling time in relation to the control samples C1 and C2. Proximity to C2 reflects the degree of recovery of the communities at the end of the experiment.

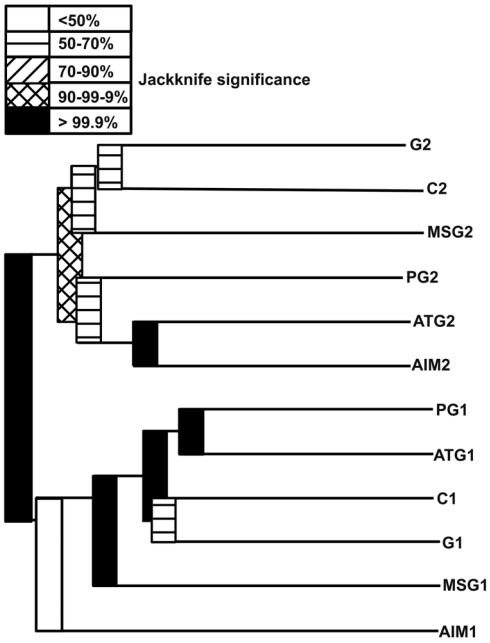

Fig. 3. Hierarchical clustering tree of the rhizobacterial communities of glyphosate-tolerant Bt-maize subject to different herbicide combination treatments.

The communities recovered from soils treated with various herbicides (C: control; G: glyphosate; PG: pethoxamid and glyphosate; MSG: mesotrione plus s-metolachlor and glyphosate; ATG: acetochlor plus terbuthylazine and glyphosate; AIM: aclonifen plus isoxaflutole and mesotrione) at two different sampling times (1: soon after emergence; 2: late before harvest) were clustered using UniFrac and subjected to jackknife statistical analysis. Branching events in the clustering tree have been tagged with their jackknife significance values according to the scale shown. Clustering separates samples first according to sampling time, and then according to the treatment applied. Community resilience to treatment is reflected in the proximity to the control at the second sampling time (C2).

according to their proximity to the control (C). At the first sampling time, the PG1 and ATG1 grouping gained a support of 100%, as did its clustering to the group formed by C1 and G1, and the grouping of their supercluster ((PG1, ATG), (C1, G1)) to MSG1. Hence, the low jackknife fraction for AIM1 was likely due to the uncertainty about the corresponding phylogenetic distance.

Samples collected at the second sampling time were further divided into two major groups (statistical support of 98%), one grouping C2 with the soils treated with G2 and MSG2, and the other clustering the rest. ATG2 and AIM2 were clustered together in 100% of the permutations. PG2 maintained an intermediate position, with the high support for the other two groupings strongly sustaining its location in the tree.

Taxonomy-Independent Alpha Diversity Analyses

The values of observed OTUs, Chao1, and Shannon index are indicated in Table 3, and the rarefaction curves of OTUs and Chao1 at the final sampling time are provided as Fig. S1.

Owing to the uneven number of reads recovered, we had to perform analyses of alpha diversity at the first and last sampling times separately, and therefore the values reported are only comparable within each sampling time but not among different sampling times. At the first sampling time, G1 and AIM1 show a decrease in their OTUs and Chao1 predicted values, while ATG1 appears significantly affected at its Chao1 predicted values.

Analysis of the values and rarefaction curves at the final

Table 3. OTU, Chao1, and Shannon index values of rhizobacterial communities subject to various herbicide treatments.

Sample[a]	OTU[b]	Chao1[c]	Shannon index[d]
Soon after emergence (4000 reads)			
C1	1,243 ± 5.01	2,245 ± 29.3	9.140 ± 0.009
G1	1,173 ± 5.16	2,126 ± 29.6	8.919 ± 0.009
PG1	1,212 ± 4.73	2,233 ± 31.1	8.968 ± 0.010
MSG1	1,221 ± 4.53	2,219 ± 33.0	9.016 ± 0.008
ATG1	1,226 ± 3.97	2,120 ± 24.2	9.054 ± 0.007
AIM1	1,121 ± 1.34	1,950 ± 12.4	9.027 ± 0.002
Late before harvest (30,000 reads)			
C2	2,989 ± 5.19	4,169 ± 24.0	9.618 ± 0.003
G2	3,095 ± 4.74	4,415 ± 20.9	9.559 ± 0.002
PG2	3,022 ± 2.28	4,046 ± 12.0	9.634 ± 0.001
MSG2	2,901 ± 2.75	3,966 ± 14.8	9.533 ± 0.001
ATG2	2,963 ± 3.92	4,089 ± 21.4	9.419 ± 0.002
AIM2	3,114 ± 3.39	4,306 ± 18.4	9.651 ± 0.001

Richness estimation in the rhizobacterial communities of Bt-maize subject to different herbicide treatments.

[a]Samples are labeled, using the initials of the treatments applied (C: control; G: glyphosate; PG: pethoxamid and glyphosate; MSG: mesotrione plus s-metolachlor and glyphosate; ATG: acetochlor plus terbuthylazine and glyphosate; AIM: aclonifen plus isoxaflutole and mesotrione) followed by the sampling time (1: soon after emergence; 2: late before harvest).

[b]OTU stands for observed taxonomic units.

[c]Chao1 provides an estimation of the actual number of OTUs in the sample.

[d]The Shannon index quantifies the entropy of the distribution and depends on the evenness of the proportional abundances of OTUs in the population. Values for the average and 95% confidence interval of the mean were computed using QIIME at a 97% similarity level and calculated from 50 replicas of 4,000 (first sampling time) and 30,000 (second sampling time) randomly selected sequence reads from each sample. Given the different sampling sizes, values are comparable within each sampling time, but not between different collection times.

sampling time obtained using more information shows that the treated soils display different degrees of resilience, as evidenced by the changes in the distance of their rarefaction curves with respect to the control.

More interestingly, evenness in the community's diversity has been suggested to be a major factor in maintaining the effectiveness of a healthy rhizobacterial community. Diversity may be evaluated using the Shannon-Wiener index, which also considers relative species abundance. Owing to the uneven sample sizes, a limited number of reads was considered, and comparisons had to be made with caution. Here, only the most extreme differences are considered. Overall, most fields showed some convergence with the control at the end of the experiment, except for ATG2, whose Shannon index remained smaller than the control over time, pointing to a loss of rare clades, and for PG2 and AIM2, which showed the opposite trend (Shannon index larger than the control at the final time), pointing to a lower weight of abundant species.

Discussion

As already mentioned, the use of maize cultivars producing Cry1Ab protein or tolerant to glyphosate has been shown to exert a minor or undetectable effect on the rhizobacterial community compared with conventional maize [4, 5, 7, 8]. The potential impact of other confounding factors was addressed by keeping all variable factors constant and pooling subsamples to address local micro-heterogeneity, as described. No appreciable differences in microbial biomass, plant growth, conditions, or yield that could induce additional rhizobacterial community changes were observed in this experiment. Microbial biomass studies usually find difficulty spotting significant differences among various herbicide treatments [30], implying that changes are compensated by differential species growth. Therefore, changes to the taxonomical composition observed in the sampling times were used to examine the progress of the rhizobacterial community.

The differences between the changes observed in the untreated soil (C) and those found in the previous studies were expected, as indicative of the different environmental conditions. Proteobacteria have been reported to be enriched normally in the maize rhizosphere [40], which is in agreement with our findings.

The soil treated solely with glyphosate (G) provides an additional baseline to contrast the combination treatments. Glyphosate [21] is a widely-used, broad-spectrum, and low-toxicity herbicide often coupled to glyphosate-resistant crops. Glyphosate is non-mobile, highly soluble, and non-persistent, which may explain why this soil revealed a reduction in alpha diversity mainly affecting Proteobacteria at the first sampling time with a full recovery of richness and diversity at the final sampling time, in agreement with previous observations in maize [17], glyphosate-tolerant maize [3, 7], and cotton [6].

The use of acetochlor plus terbuthylazine combined with the post-emergence treatment with glyphosate (ATG) after a comparison with the C and G samples confirms this treatment as being more distant from the control, hence exerting a greater impact that affects Proteobacteria to a wide extent. Acetochlor is moderately soluble and non-persistent, and is suspected to have endocrine and carcinogenic effects in humans, amphibians, and fish [20].

Like racemic metolachlor, it has been reported to affect antioxidant enzymes in soil bacteria [36]. Residual acetochlor effects on the rhizosphere have recently been reported to be detectable soon after application (until day 15), but is very low afterwards [2]. Terbuthylazine has algaecide, herbicide, and microbiocide properties and is primarily adsorbed by organic matter in the soil, with the bacterial community playing a leading role in its degradation. It has been reported to significantly alter soil bacterial composition by increasing denitrifying bacteria in soils, possibly reducing the efficiency of fertilizers [31]. The reduced effect of glyphosate and acetochlor when used alone suggests that the toxic degradation products, low solubility, and moderate persistence of terbuthylazine could be responsible for the deep and lasting effect of this combination on the rhizobacterial community.

The combination of pethoxamid and glyphosate (PG) exerted a significant early impact and a relevant late impact on the community, although it seemed to be less aggressive than the treatment with acetochlor and terbuthylazine plus glyphosate. Pethoxamid was reported as a selective systemic herbicide [32] for maize and soya bean that is absorbed by the roots and young shoots. Since glyphosate is non-persistent and pethoxamid is a moderately soluble non-persistent herbicide, the presence of lasting effects suggests a synergy where the early effects observed may be dramatic enough to outlast the herbicide presence in the soil.

Pre-emergence treatment with s-metolachlor and mesotrione followed by post-emergence treatment with glyphosate (MSG) exerted a considerable early impact that was largely restored at the final sampling time. Mesotrione was developed to control broad leaf weeds in maize crops [29] and is reportedly degraded rapidly by soil microorganisms; maize is naturally tolerant to mesotrione with no yield penalty. Mesotrione has moderate solubility and persistence and can be degraded by soil bacteria (*Bacillus* sp.) [9]. The effect of mesotrione has been studied using polymerase chain reaction temporal temperature gradient gel electrophoresis 16S rDNA fingerprints [12], showing no response from the microbial communities at field rate. The biological activity of s-metolachlor was discovered in 1970, and it is still widely used on account of its high efficiency [45], although it is becoming less common owing to its bioaccumulation and potential carcinogenic, cytotoxic, and genotoxic effects. S-Metolachlor has been reported to influence *Pseudomonas* sp. metabolism and to exert high toxicity on microalgae species [45], but is less persistent. The combined synergistic effects of mesotrione and s-metolachlor on rhizobacteria have been the subject of recent analysis by polymerase chain reaction denaturing gradient gel electrophoresis [30], showing a synergistic effect equivalent to a 10-fold field rate. Our finer-grained analyses indicate that despite a strong initial effect, this combination shows good resilience by the convergence of its rhizobacterial community composition with that of the control at the end of the study.

The combination of aclonifen with isoxaflutole has been reported to exert no depressive effect on maize yield [39]. We have tested the impact of aclonifen plus isoxaflutole followed by mesotrione (AIM) on rhizobacteria. The observed effects might be related to the low solubility, moderate mobility, and low persistence of isoxaflutole and to the persistence and low solubility of aclonifen, which is known to affect carbon mineralization microorganisms in sand and loam and to affect bean yield *via* its effects on *Rhizobium phaseolii* [23]. Isoxaflutole is known to increase nutrient level reduction in soils [34], and the effect of its combination with atrazine has been compared to that of glyphosate alone [26] without any major differences. Aclonifen is used in conjunction with isoxaflutole (an isoxazole) in pre-emergence application to inhibit the growth of grasses and broad leaf weeds.

Our findings in the treatment with a different combination (isoxafutole plus aclonifen followed by mesotrione) further suggest that the effect may be due mainly to the presence of aclonifen, and possibly, to a much lower extent, mesotrione and isoxaflutole.

Verrucomicrobia seems to be the phylum that is less affected, usually filling the void left by other phyla, and suggesting that it may have a relevant opportunistic or compensatory role in culture soil. Proteobacteria are mostly affected at the end of the ATG treatment and Acidobacteria at the end of the PG treatment. Changes observed at lower taxonomical levels, although potentially relevant [16], are difficult to assess owing to the uneven sample sizes.

The extent to which the rhizobacterial composition from each sample differs at the final time is indicative of the long-term effects of each treatment on rhizobacterial composition. All the analyses performed (taxonomical, phylogenetic, and alpha and beta diversities) support the separation of samples into two main groups at the end of the experiment; one composed of samples C2, G2, and MSG2, and a second one consisting of samples PG2, AIM2, and ATG2, with PG2 taking an intermediate position. This separation is strongly supported by the statistical analyses.

In conclusion, from all the treatments considered, the use of glyphosate alone to treat glyphosate-tolerant maize seems the less aggressive one for the rhizobacterial community. The combination of the pre-emergence treatment

with mesotrione and s-metolachlor followed by the post-emergence treatment with glyphosate shows a good final resilience. The effect of pethoxamide and glyphosate displays lasting effects on rhizobacteria and seems less aggressive than using a treatment suitable for conventional non-glyphosate-tolerant maize with aclonifen and isoxaflutole followed by mesotrione. The treatment with acetochlor plus terbuthylazine in pre-emergence, followed by glyphosate in post-emergence, exerts a great impact, which is in agreement with previous observations. We must emphasize, however, that this study has been conducted in a single location over a one-year cultivation period. Our observations support the need for more in-depth analyses of soil microbial properties (such as functional microarrays) and for spreading the analyses to other locations and over several years of cultivation, so as to accurately measure the cumulative effects of the repeated application of each treatment, leading to sounder recommendations for long-term agricultural practices.

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