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# Glyphosate affects the rhizobacterial communities in glyphosate-tolerant cotton

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ABSTRACT

The use of herbicides to kill undesirable weeds is an important element of agricultural management that can greatly alter soil characteristics. Moreover, the composition of rhizobacterial communities varies according to the soil texture. The effect of glyphosate, a post-emergence applied herbicide, on the rhizobacterial communities of genetically modified GHB614, a glyphosate-tolerant cotton, was evaluated in two different agricultural fields, one with clayey soil and the other with clayey-loam soil texture. The potential effect was monitored at two different sampling times (7 days after glyphosate application and just before crop harvesting) by high throughput DNA pyrosequencing of rhizobacterial DNA coding for the 16SrRNA hypervariable V6 region. The taxonomic analysis indicated that *Proteobacteria*, *Acidobacteria* and *Actinobacteria* were the more abundant taxa in both fields, although the UniFrac phylogenetic analysis differentiated one field from the other. To analyse bacterial diversity, MUSCLE alignment, DNADIST distance calculation and Mothur clustering were compared with the ESPRIT software package and both approaches gave consistent results. Thus, rhizobacterial diversity was apparently higher in the clayey soil than in the clayey-loam, judging from the OTUs and diversity index estimates. The glyphosate treatment, in general, does not seem to greatly affect the structure of bacterial communities in the cotton rhizosphere. However, the degree of recovery of the soil bacterial communities throughout plant growth was apparently less effective in the clayey-loam field than in the clayey one, strongly suggesting that recovery does indeed greatly depend on the soil textures and their associated bacterial community diversity.



## 1. Introduction

Soil bacterial communities are an essential component of its quality and fertility (Gans et al., 2005). On the other hand, agricultural management intensity can alter soil microbial community structure (Jangid et al., 2008). In this context, it is relevant to measure the potential effects of herbicides on agricultural soil microbial communities and, in particular, the effect of broad-spectrum herbicides to control the growth of undesirable weeds. Glyphosate (2-(phosphonomethyl) glycine) is a broad-spectrum systemic herbicide that is used as a weed control in glyphosate-tolerant crops where genetically modified herbicide-tolerant plants are grown. These herbicide-tolerant cultivars include plants such as soybean, corn and cotton, in decreasing order of cultivated surface (Funke et al., 2006, 2009; Kremer and Means, 2009; Lundgren et al., 2009; Sidhu et al., 2000). In particular, cotton is considered to be one of the most important crops for world consumption (Chapagain et al., 2006).

It is known that glyphosate absorbed in the leaves of glyphosate resistant plants can alter root exudation and hence affect rhizosphere communities (Kremer et al., 2005). The influence of glyphosate on soil microorganisms from different cultivars has been reviewed (Cerdeira and Duke, 2006; Locke et al., 2008; Kremer and Means, 2009). In particular, glyphosate inhibits the growth of certain soil bacterial species (Gimsing et al., 2004; Liu et al., 1991) and may also favour colonization of soilborne fungi (Descalzo et al., 1998; Lévesque and Rahe, 1992), although, eventually, it may trigger an increase in total soil microbial activity, due to the ability of certain bacteria to metabolize glyphosate (Mijangos et al., 2009).

Culture-independent nucleic acid-based methods have been used to characterize microbial communities (Amann et al., 1995). Next generation sequencing of the hypervariable V6 region of the 16S rRNA gene has been shown to be a powerful and effective tool for studying the bacterial composition of several habitats (Sogin et al., 2006; Huber et al., 2007; Huse et al., 2008), including forest and agricultural soils (Roesch et al., 2007; Acosta-Martinez et al., 2010). The use of this technique to evaluate the herbicide effect on maize rhizobacterial communities has shown that herbicides do affect rhizobacterial community structure and that

*Abbreviations:* GMHT, genetically modified herbicide-tolerant; SSU rRNA, small subunit ribosomal RNA; EPSPS enzyme, 5-enolpyruvylshikimate-3-phosphate synthase; OTUs, operational taxonomic units.

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the post-emergence applied glyphosate was environmentally less aggressive than the pre-emergence applied herbicide, *Actinobacteria* being the bacterial group most affected by both herbicides (Barriuso et al., 2010).

Therefore, it is of great interest to determine the potential negative effect that the use of glyphosate may have on agricultural soil bacterial communities of glyphosate-tolerant plants, as the composition of rhizobacterial communities varies with soil texture (Fang et al., 2005; Val et al., 2009). We have made use of massive parallel sequencing of the SSU rDNA V6 hypervariable region to assess the potential environmental effect that the use of glyphosate applied as a post-emergence herbicide may have on GHB614 glyphosate-tolerant cotton in two different agricultural soils. The objectives of this study were: (1) to compare the soil bacterial community under two different soils as affected by glyphosate herbicide applications to glyphosate tolerant-cotton and (2) to compare the diversity analysis from the pyrosequencing data by using two different approaches. To accomplish this, the structure of the bacterial communities was taxonomically analysed, the phylogenetic distances estimated and the bacterial diversity analysed using two different methods.

## 2. Materials and methods

### 2.1. Plant material and sampling

Glyphosate-tolerant cotton (*Gossypium hirsutum* L.), event GHB614, variety BBS-AS-GT, containing a glyphosate-tolerant EPSPS enzyme (5-enolpyruvylshikimate-3-phosphate synthase) double-mutated from *Zea mays*, was grown in two commercial cotton fields. Field 1 was located in Coria del Rio (N 37°17′44″, W 06°00′02″; Sevilla, Spain) and field 2 was located in Dos Hermanas (N 37°14′57″, W 05°56′12″; Sevilla, Spain) and the experiments were carried out during development throughout 2009 and 2010, respectively. According to EU regulations on the experimental cultivation of genetically modified plants not yet authorized, a field used one year cannot be seeded the next, so that monitoring of eventual re-emergent modified plants can be conducted. Therefore, field 2 was selected as the closest possible agricultural soil available. The climatology was similar for both fields. The accumulated precipitation in the cultivation period (May–September) in the Sevilla region was 30.9 mm in 2009 and 27.9 mm in 2010 (http://clima.meteored.com). Potential climatological differences have been ruled out, as the precipitation levels were similar in the two fields. In both cases, an equivalent number of plants were treated in post-emergence with glyphosate (GLYPHOS®, containing glyphosate as isopropylamine salt, 360 g $L^{-1}$; 3 L $ha^{-1}$) or not treated with herbicide at all (control soil). The surface of each experimental field measured 40 $m^2$. Cotton GHB614 seeds were obtained from Bayer CropScience. No special weed control method was followed, as the untreated soil was to serve as a control of weed proliferation. Glyphosate was applied when the plants were approximately 8–10 cm tall. Plants were harvested from each soil at two different growth stages: 7 days after glyphosate application (first sampling time) and just before crop harvesting at final growth, approximately six months after seeding (final sampling time). Roots and adhered soil were collected. The term "rhizosphere" describes the carefully separated soil adhered to these roots. Given the small size of the cotton fields these were divided into 4 randomized blocks, and six samples were taken from each block at the time of collection so that a total of 24 samples were collected from each treatment at every collection time, and an equal amount of soil from each sample was pooled for the subsequent analyses in all cases. This methodology has been used before for the analysis of experimental fields of genetically modified plants (Barriuso et al., 2010, 2011a,b).

### 2.2. Soil texture

Field 1 has a clayey soil (51% clay, 29% silt and 20% sand) with 1.21% OM, CEC 11.08 meq per 100 g and an electrical conductivity of 519 μ $cm^{-1}$, while field 2 has a clayey-loam soil (35% clay, 25% silt and 40% sand) with 1.66% OM, CEC 8.85 meq per 100 g and an electrical conductivity of 570 μ $cm^{-1}$ (determined by Agriquem S.L. in Seville, Spain). Soils from both fields are Mediterranean Xeralfic Alfisols. Adsorption coefficient values (Kd values) were estimated based on Koc values (Koc = 100Kd/%OC; Sluszny et al., 1999), as detailed in the PPDB (pesticide properties data base web site, http://sitem.herts.ac.uk/aeru/footprint/). Considering that %OC = %OM/1.7, estimated glyphosate Kd values were 154.44 and 211.88 for fields 1 and 2, respectively.

To determine the chemical properties of the soil, rhizosphere samples were ground using a mortar and dried at 60 °C for 12 h. Total mean values for carbon and nitrogen soil content from three independent measures were determined with a Leco CHSN-932 autoanalyser (Leco, Düsseldorf, Germany). The chemical properties of the soils showed carbon values of 3.06% and 2.95%, and nitrogen values of 0.14% and 0.13% for fields 1 and 2, respectively.

### 2.3. DNA extraction, PCR amplification of the V6 16S rRNA gene and pyrosequencing

Rhizospheres from the different collection times were pooled and three independent DNA extractions each from one gram of soil performed using the PowerMax Soil DNA kits (MO Bio Laboratories Inc., USA) following instructions from the supplier. Soil DNA from each of the three independent extractions was used as template for PCR amplification of the V6 hypervariable region of the 16S rDNA. The oligonucleotide design included 454 Titanium Life Science's A (5′-CGTATCGCCTCCCTCGCGCCATCAG-3′) or B (5′-CTATGCGCCTTGCCAGCCCGCTCAG-3′) pyrosequencing adaptors fused to the 5′ end of the template specific primers: 967F (5′-CAACGCGAAGAACCTTACC-3′) and 1046R (5′-CGACAGCCATGCANCACCT-3′), where a MID (Multiplex Identifier) was included immediately preceding the V6 specific primer, so that samples from the two soils at the three sampling times could be analysed in a single lane of the 454 Titanium pyrosequencer. The obtained sequences were deposited in the NCBI sequence reads archives (accession number SRA009281). For field 1, the MIDs used in each 454 plate at the first sampling time were MID9 (5′-TAGTATCAGC-3′ and 9106 sequences were obtained) for the untreated soil and MID10 (5′-TCTCTATGCG-3′ and 7216 sequences were obtained) for the glyphosate-treated soil, and at the final sampling time, MID12 (5′-TACTGAGCTA-3′ and 13 049 sequences were obtained) for the untreated soil, MID13 (5′-CATAGTAGTG-3′ and 19 687 sequences were obtained) for the glyphosate treated soil. For field 2 the MIDs used at the first sampling time were MID4 (5′-AGCACTGTAG-3′ and 4155 sequences were obtained) for the untreated soil and MID5 (5′-ATCAGACACG-3′ and 3099 sequences were obtained) for the glyphosate-treated soil, and at the final sampling time, MID7 (5′-CGTGTCTCTA-3′ and 4162 sequences were obtained) for the untreated soil and MID8 (5′-CTCGCGTGTC-3′ and 3777 sequences were obtained) for the glyphosate-treated soil. Soil DNA was used as template for PCR amplification by incubation at 95 °C for 5 min, followed by 30 cycles of incubation at 95 °C (30 s), 63 °C (45 s) and 72 °C (1 min), with a final extension cycle of 5 min at 72 °C. The amplified DNA resulting from the three independent PCR reactions for each DNA template preparation was pooled and cleaned (Illustra GFX PCR DNA purification kit, GE Healthcare), checked with the Bioanalyser 2100 (Agilent technologies), quantified with Quant-IT-picogreen (Invitrogen) and used to make the single strands on beads, as required for 454 pyrosequencing (Margulies et al., 2005). Pyrosequencing was carried out using a