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Increased microbial activity and nitrogen mineralization coupled to changes in microbial community structure in the rhizosphere of Bt corn

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ABSTRACT

The interactions between plant roots and soil microorganisms are essential for the function and stability of ecosystems, primary agricultural production and plant health. Despite the importance of soil microbes the response of these microbes to large-scale cultivation of genetically modified (GM) crops is still poorly understood. This study evaluated the potential impact of two lines of transgenic Bt maize on rhizosphere microorganisms. A time-course field experiment was conducted over a period of two years in two fields in Guadalajara (Spain) with monthly sampling from April to September. Rhizosphere soil was collected from transgenic (TG) and unmodified (WT) maize plants from each field and sampling time for the analysis of several important functional and structural soil quality parameters. Total microbial activity, as determined by H^3-Thymidine and C^{14}-Leucine incorporation, was found to be higher in the rhizospheres of the transgenic plants. Similarly, differences in potential ammonification and nitrification were observed in the second year of the study. In contrast, bacterial and fungal microbial catabolic abilities, as determined by Biolog ECO and FF plate analyses, respectively, were more influenced by sampling time than the transgenic nature of the plants. Microbial community structure was also studied by bacterial and phylum-specific PCR-DGGE and PCR cloning approaches. In general, differences were again more pronounced between sampling times, as opposed to between TG *versus* WT plants, although marked differences were observed within the *Betaproteobacteria* between plant lines. For the first time it describes the presence of *Iamiaceae* family in soil, specifically to TG plant rhizosphere. To summarize, the study showed that some important properties of rhizopshere microbes may be impacted by Bt maize cultivation and highlighted the fact that such potential effects need to be viewed within the context of seasonal and spatial variability.

1. Introduction

The use of genetically modified (GM) plants has a great potential for agriculture. Some of the most important of these modified crops are varieties that incorporate insecticidal *cry* genes from *Bacillus thuringiens* (Bt plants) to provide protection against crop-damaging insect pests. This strategy can reduce problems associated with the use of broad-spectrum chemical pesticides, since the toxins are produced continuously within the plants and exhibit relatively high specificity for insect pests (Flores et al., 2005). These advantages have led to the increased cultivation of Bt transgenic crops, and Bt maize is, by far, the most widely grown Bt crop in the world. Six European countries planted 91,193 ha of Bt maize in 2010, led by Spain (James, 2010). Nevertheless, concerns remain that there may be potential risks or unwanted effects associated with Bt crop cultivation not only above ground, but also belowground. Although the impact of Bt crops on soil ecosystems has been studied for over 15 years (Donegan et al., 1995; Icoz and Stotzky, 2008), data is still limited with respect to the potential influence on soil-borne microbial communities and functions.

Microorganisms are the dominant soil organisms, both in terms of biomass and activity. In soil, they are involved in numerous important processes, including decomposition of organic matter, nutrient mineralization, regulation of plant pathogens, decomposition of agricultural chemicals, and improvement of soil structure (Bruinsma et al., 2003; Gupta and Yeates, 1997). Given these

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important functions and the potential for close interaction with transgenic plants, there is a potential that some soil microorganisms might inadvertently be affected by the Cry proteins released from Bt crops, potentially leading to altered or disturbed soil communities (Kowalchuk et al., 2003).

Microbial densities and activities are stimulated in the rhizosphere (Bowen and Rovira, 1999), and plant-microbe interactions in the rhizosphere regulate numerous aspects of plant health and growth. The quality and quantity of root exudates and residual plant materials can have an important impact on the organisms residing in the rhizosphere (Bardgett et al., 1999; Kowalchuk et al., 2002). Therefore, any change in the quality and quantity of root exudates could potentially modify the composition and activity of the soil microbiota and may cause changes in both deleterious and beneficial microorganisms. In this sense, it is well known that Bt toxins may enter soil by incorporation of plant residues after harvest of a Bt crop (Stotzky, 2004; Tapp and Stotzky, 1998) or *via* the root exudation of Bt plants (Saxena et al., 2002, 2004). However, the residence time of Cry proteins in soil is a subject of debate, with some studies suggesting that Cry proteins can remain in soil for months or years (Saxena and Stotzky, 2002; Stotzky, 2004), yet others indicating a much more transient presence, on the order of days (Palm et al., 1996; Sims and Ream, 1997).

The present study has been carried out on two Monsanto's YieldGards corn lines, expressing the Cry1Ab protein, which is the most widely grown Bt crop today (Benedict and Ring, 2004). The objective of this study was to examine the responses of rhizosphere microbial communities in response to Bt corn cultivation. The study focussed on potential effects on microbial community structure, activity, metabolic abilities and potential nitrogen transformations. It included transgenic and unmodified maize plants, grown in two different fields over the course of two years. Combination of these variables allowed us to examine the impact of transgenic plants on soil microbial communities within the context of seasonal variability.

Two distinct approaches to examine the potential impact of transgenic maize on microbial communities in the rhizosphere were taken. From a functional perspective, metabolic abilities of rhizosphere microorganisms using Biolog plates (Garland and Mills, 1991), nitrogen transformations and microbial activity (*via* potential respiration thymidine and leucine incorporation methods) were studied. From a structural perspective, microbial community structure was studied. Bacterial, fungal and group-specific PCR-DGGE and bacterial 16S rRNA gene clone library were analysed.

2. Materials and methods

2.1. Plant lines and field sites

Rhizosphere samples were collected from two 1 ha commercial crop fields located in Guadalajara (Spain), separated from each other by approximately 12 km: Marchamalo (40°40′14″N; 3°12′2″W), and Yunquera de Henares (40°45′19″N; 3°9′5″W). Following the soil classification of USDA 1987, these soils were: Order Inceptisol, Group Xerochrept, Association Xerochrept/Xerothent. These two fields were used for agronomic production, not experimental plots. Half of each field was sown with transgenic maize (TG) and the other half with unmodified maize (WT). According to the national legislation, transgenic and unmodified plants cannot be arranged randomly (mixed), and plots sown with TG maize must be surrounded by 2 m of unmodified maize as a protective measure against potential problems of resistance.

The two transgenic maize Bt plant lines used derived from the unmodified lines PR33P66 and Tietar, which are very similar varieties. Both transgenic lines were obtained by transforming PR33P66 and Tietar with the same event MON810, which express Cry 1 Ab protein providing PR33P67 and DKc 6575 transgenic lines respectively. PR33P66 (WT) and PR33P67 (TG) were grown in Marchamalo. Tietar (WT) and DKc 6575 (TG) were cultivated in Yunquera de Henares, and each field was considered a replicate. All seeds were provided by Monsanto Agricultura España S.L.

Before sowing, representative physicochemical analysis (pH, CO, ammonium, nitrate, total nitrogen) was carried out and the homogeneity between the two parts (TG and WT) within each field was verified.

In each field, each part (TG and WT) was divided into 10 m × 10 m plots, which were numerated. Using a software, random numbers were generated and 9 plots were selected in each part: 9 in TG and 9 in WT. In each plot, the soil closely adhered to roots (rhizosphere soil) of one maize plant was sampled, the rhizosphere from 3 plants were pooled and thus constituted a repetition. Therefore, for each plant (TG and WT) there were two replicates (one per field), with three repetitions each, for two consecutive years.

Samples were taken once a month during the maize phenological cycle (from April to September, with the exception of August) in two consecutive years (2004 and 2005). The first sampling in each year (T0 and T5) was done before sowing (April), in the absence of plants and only one set of triplicate was taken (coded as TG-WT). Both years of experiment TG and unmodified (WT) plants were sown in the same part of each field. Agricultural management performed in each field was the same both years, and weather conditions similar, allowing us to consider the year as a variable. Moreover, for the first time in 2004, when the study started, TG maize was sown in both fields. Therefore, it was very interesting to see if the effects found in the first year, were maintained or increased with a second year of crop.

2.2. Physico-chemical analysis

Soil organic carbon and pH were determined for each replicate and repetition at each sampling time. Organic carbon was determined by weighing 2 g of soil (previously dried at 105 °C), and maintaining it at 400 °C in a muffle furnace Hobersal JB-20 for 4 h. During this time, organic matter was burned and the sample was removed from the oven and put into a desiccator to exclude moisture and air humidity and was reweighed. The weight difference expressed as a percentage represents the content of organic matter (Forster, 1995).

Ammonium (Weatherburn, 1967), and nitrate (Nitrate-test kit; Merck) contents were determined as described below for the potential ammonification and potential nitrification assays.

2.3. Analysis of nitrogen transformations in the rhizosphere and potential soil respiration

The determination of the potential free nitrogen fixation, potential denitrification and potential soil respiration were evaluated under laboratory conditions. Briefly, 2.5% glucose and water were added to 100 g of soil until maximum retention capacity (potential conditions) was reached. This was incubated in leakproof containers of 600 ml (in which 10% of the atmosphere was replaced by acetylene) at room temperature for 96 h. Ethylene, nitrous oxide and CO_2 produced were evaluated using a KNK-3000-HRGC gas chromatograph (Konik Instruments, Barcelona, Spain) equipped with a thermal conductivity detector (TCD) and a Chromosorb-101 column (80/100 mesh) 200 cm long and 0.2 cm in diameter under the following conditions: Column temperature: 40 °C; Injector temperature: 50 °C; Detector temperature: 100 °C; Carrier gas: Helium; Carrier gas flow rate: 20 ml/min; Sample volume: 1 ml. Under these conditions ethylene, nitrous oxide and CO_2 can be