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A two-year field study with transgenic *Bacillus thuringiensis* maize: Effects on soil microorganisms

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ARTICLE INFO

Article history:
Received 30 October 2007
Received in revised form 4 April 2008
Accepted 30 May 2008
Available online 24 July 2008

Keywords:
Bt maize
Culturable soil microbiota
Dehydrogenase activity
Nitrogenase activity
ATP content

ABSTRACT

We evaluated the changes of some soil microbiological characteristics due to the use of transgenic maize expressing *Bacillus thuringiensis* (*Bt*) toxin. A two-year field experiment was conducted (2003 and 2004). Two lines of transgenic *Bt* maize that express the Cry1Ab protein (event 176 and MON 810) and their near-isogenic non-*Bt* lines were used. Rhizosphere and non-rhizosphere soils were collected and measurements were performed during the maize cultural cycle and immediately at pre-harvest. Key soil microbiological parameters measured included the numbers of culturable aerobic bacteria, including actinomycetes, and fungi, the activity of dehydrogenase and nitrogenase enzymes and ATP content.
There were clear seasonal effects in the microbial parameters as evidenced by the consistent changes in sampling dates across the two years. Differences in the measured variables were also observed between rhizosphere and non-rhizosphere soils. However, under our field conditions, the presence of *Bt* maize did not cause, in a general way, changes in the microbial populations of the soil or in the activity of the microbial community.

1. Introduction

The insertion into plants of genes from *Bacillus thuringiensis* (*Bt*) that code for the production of insecticidal toxins (Cry proteins) reduces many problems associated with the use of chemical pesticides, as the toxins are produced continuously within these plants. However, the insecticidal protein, Cry 1Ab, from *Bt* maize is introduced into soil primarily in root exudates (Saxena et al., 1999; Saxena and Stotzky, 2000) and also by incorporating plant residues after harvesting the crops (Tapp and Stotzky, 1998; Zwahlen et al., 2003). *In vitro* and *in situ* studies indicated that the Cry 1Ab protein was also present in the rhizosphere soil of field-grown *Bt* maize plants throughout their growth and several months after their death (Saxena and Stotzky, 2000). Although most *Bt* Cry proteins have high specificity, their effects on non-target organisms have not been fully evaluated.

Under laboratory conditions, no effect of the Cry1Ab protein was found on collembolans (Sims and Martin, 1997; Heckmann et al., 2006), isopods (Escher et al., 2000), protozoa, nematodes, fungi, bacteria, algae, or earthworms (Saxena and Stotzky, 2001; Koskella and Stotzky, 2002; Baumgarte and Tebbe, 2005; Vercesi et al., 2006).

Other studies have examined the effects of *Bt* crops on soil ecosystem functions, such as residue decomposition. Most of these studies have compared the decomposition of *Bt* and non-*Bt* plant residues. Hopkins and Gregorich (2003) did not observe any detectable difference in the decomposition of plant material from *Bt* and non-*Bt* maize. Accordingly, Flores et al. (2005) found that numbers of culturable bacteria and fungi and the activities of representative enzymes involved in the degradation of plant biomass were not different between unamended soil or amended with biomass of *Bt* and non-*Bt* plants.

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doi:10.1016/j.scitotenv.2008.05.046

Some detrimental effects, such as mortality and reduced fecundity, have been observed in non-target invertebrates exposed to various *Bt*-producing strains (Mulla et al., 1982; Flexner et al., 1986). Studies of soil microbial and microfaunal communities also revealed differences in bacterial and fungal CLPP profiles (Blackwood and Buyer, 2004) and in nematodes population (Griffiths et al., 2005) under *Bt* maize cultivation. Donegan et al. (1995) observed effects of transgenic *Bt* cotton on both abundance and diversity of indigenous soil bacteria and fungi.

Biological and biochemical properties of soil have often been proposed as early and sensitive indicators of soil ecological stress or other environmental changes (Visser and Parkinson, 1992; Dick, 1994; Oliveira and Pampulha, 2006). Measurement of microbial populations in combination with their activity provides more sensitive information of the microbial changes than either activities or population analysis alone (Brookes, 1995).

Nitrogen fixation is performed by phylogenetically and physiologically diverse groups of prokaryotic organisms and is rapidly affected, positively or negatively, when environmental conditions are changed (Mårtensson, 1993). Dehydrogenase activity (DHA) and ATP content are also widely used in evaluating the metabolic activity of soil microorganisms (Bastida et al., 2007; Crecchio et al., 2007; Tejada et al., 2008).

The aim of this research was to evaluate changes in the soil microbiota due to the use of transgenic *Bt* maize. We performed a two-year experiment under field conditions with two hybrids of transgenic *Bt* maize and their near-isogenic lines. Soil microbiological parameters measured included numbers of culturable aerobic bacteria, including actinomycetes, and fungi, the activity of dehydrogenase and nitrogenase enzymes, and ATP content.

2. Materials and methods

2.1. Site and plants

The study was carried out on an experimental farm in the central region of Portugal. The soil is a well-drained sandy soil (pH, 6.2; organic carbon, 12 g kg^{-1}; sand, 80.2%; silt, 18.6%; clay, 1.3%). The experiment was conducted in two successive years (2003 and 2004) in a large plot (160 m × 60 m) where transgenic *Bt* or non-*Bt* maize had never been planted. This plot was subdivided into 4 sub-plots (80 m × 30 m each). Two varieties of transgenic *Bt* maize [CG 00256-176, Cry1Ab (Compa Cb), from Syngenta and MON 810, Cry 1Ab (Elgina), from Pioneer] and their near-isogenic lines (Dracma and Cecilia, respectively) were used in this study. Each sub-plot was sown with a different maize line, under irrigated conditions. Soils were sampled from the 4 sub-plots, between 0 and 20 cm deep. The sub-plots were established in 2003 as part of a larger study examining the efficiency of the utilization of *Bt* maize in the control of *Sesamia nonagrioides* and *Ostrinia nubilalis*.

2.2. Soil sampling

Rhizosphere and non-rhizosphere soil samples were collected, in both years, 30 days after sowing (2003: middle June; 2004: beginning of June) and immediately at pre-harvest (2003: beginning of October; 2004: end of September).

Ten soil samples were collected from each sub-plot (5 rhizosphere samples and 5 non-rhizosphere samples). Non-rhizosphere soil was taken at a depth of 0–20 cm. To obtain rhizosphere soil, root material with adhering soil was removed with a trowel and placed in a plastic bag. All visible plant debris was removed manually. Each soil sample was then sieved (2 mm), and stored at 4 °C, in the dark, before analysis.

2.3. Enumeration of bacteria, actinomycetes and saprophytic fungi

Colony-forming units (CFU) of culturable heterotrophic bacteria, actinomycetes, and fungi, were determined by serial dilution and plating on selective media. Serial dilutions of soil samples (1 g fresh weight) were made with 1/4 strength Ringers solution. Plate counts of culturally-viable bacteria were made on Tryptone Soya Agar (TSA, Oxoid) amended with 0.1 g of cycloheximide l^{-1}. For fungi, the medium was Rose Bengal Agar (RB, Oxoid) amended with 30 mg of streptomycin sulphate l^{-1}. Actinomycetes were counted on Glycerol Casein Agar (Williams and Wellington, 1982) amended with 0.05 g of cycloheximide l^{-1}. The plates were inoculated with 0.1 ml of soil suspension and incubated at 25 °C for 4–7 days for fungi and heterotrophic bacteria and for 10 days for actinomycetes. All the results are expressed on an oven-dry wt basis.

2.4. Dehydrogenase activity

Dehydrogenase activity (DHA) was determined by the method of Tabatabai (1982) using 1 ml of a 3% triphenyl tetrazolium chloride (TTC) solution per 20 g of soil (dry weight equivalent).

Table 1 – Example ANOVA for bacteria data

Source of variation	Degrees of freedom	Sum of squares	Mean squares	*F*	Probability
Year (Y)	1	9211.2	9211.2	201.4	0.0008
Maize (M)	3	457.9	152.6	3.3	0.1743
Year × maize	3	137.2	45.7	6.3	0.0005
Sample date (Y × M)	8	1197.7	149.7	2.7	0.0897
Rhizosphere/non-rhizosphere soil (Y × M)	8	3975.6	497.0	9.0	0.0027
Sample date (Y × M) × rhizosphere/non-r (Y × M)	8	441.2	55.2	7.6	0.0000
Error	128	928.7	7.3		
Total	159				

Significant sources of variation at the 0.05 probability level are in bold font.

Table 2 – Effects of *Bt* maize on heterotrophic aerobic bacteria (mean CFU × 10^6 g^{-1} dry soil)

	Elgina (t)	Cecilia	Compa (t)	Dracma	Average
Sowing	18.94	15.95	11.33	11.12	14.33
Rhizosphere	23.70	20.65	13.70	13.52	**17.89**
Non-rhizosphere	14.17	11.25	8.95	8.71	**10.77**
Harvest	15.94	18.45	17.66	15.94	16.99
Rhizosphere	20.43	26.58	22.93	21.80	**22.94**
Non-rhizosphere	11.44	10.32	12.38	10.07	**11.05**
Average	**17.44**	**17.20**	**14.49**	**13.53**	15.66
Tukey's HSD	a	a	b	b	

(t) = transgenic line. Sowing and Harvest refer to the two sampling times, while Rhizosphere and Non-rhizosphere refer to the two soil sampling areas, which were nested within the sampling times. Year is not presented because it is a block term. Marginal averages in column 6 in bold font were significantly different as determined by ANOVA, while those in the last row were determined by Tukey's HSD test. In the latter, means followed by different letters are significantly different at the 0.05 probability level.

TTC is converted to triphenyl formazan (TPF), a red dye that is detected using a spectrophotometer (485 nm) after incubation (24 h at 37 °C).

Results were expressed in μg TPF · g^{-1} of dry soil 24 h^{-1} and were calculated from spectrophotometer calibration in the range of 0–500 μg TPF · g^{-1} of dry soil.

2.5. *Nitrogenase activity*

Nitrogenase activity was measured as acetylene reduction activity (ARA). Soil samples corresponding to 20 g of dry weight were placed in serum bottles, and 5 ml of Combinated Carbon (CC) medium (Rennie, 1981) was added. After a pre-incubation period of 24 h at 25 °C, samples were incubated for 24 h at 25 °C with 10% (vv^{-1}) acetylene (C_2H_2). Gas samples were obtained from the bottles with gas-tight syringes and analysed for ethylene (C_2H_4) using a gas chromatograph Varian 3800GC (Varian Analytical Instruments, Mitchell Drive, Walnut Creek, USA), fitted with a 1 m × 1/8″ column packed with Porapak T(80–100 mesh) and a flame-ionization detector (FID). Corrections were made for traces of C_2H_4 in the C_2H_2. The results, the means of 10 replicate samples, were expressed on the basis of dry weight of soil.

Table 3 – Effects of *Bt* maize on actinomycetes (mean CFU × 10^5 g^{-1} dry soil)

	Elgina (t)	Cecilia	Compa (t)	Dracma	Average
Sowing	11.47	14.90	8.63	8.84	**10.96**
Rhizosphere	9.74	13.35	6.35	7.70	9.29
Non-rhizosphere	13.19	16.44	10.91	9.97	12.63
Harvest	21.70	21.75	23.50	21.60	**22.14**
Rhizosphere	24.30	24.80	23.80	21.10	23.50
Non-rhizosphere	19.10	18.70	23.20	22.10	20.78
Average	**16.58**	**18.32**	**16.07**	**15.22**	16.55
Tukey's HSD	b	a	b	b	

(t) = transgenic line. See Table 2 for explanation.

Table 4 – Effects of *Bt* maize on fungi (mean CFU × 10^4 g^{-1} dry soil)

	Elgina (t)	Cecilia	Compa (t)	Dracma	Average
Sowing	12.39	8.27	10.40	13.40	11.12
Rhizosphere	10.14	6.70	8.50	11.75	9.27
Non-rhizosphere	14.64	9.84	12.30	15.05	12.96
Harvest	22.42	21.38	13.57	14.29	17.91
Rhizosphere	30.08	27.09	9.62	11.45	19.56
Non-rhizosphere	14.76	15.67	17.51	17.13	16.27
Average	**17.41**	**14.83**	**11.98**	**13.85**	14.51
Tukey's HSD	a	b	c	b	

(t) = transgenic line. See Table 2 for explanation.

2.6. *ATP content*

The ATP content in soil was determined according to published methods (Oades and Jenkinson, 1979; Tate and Jenkinson, 1982), with some modifications. Fresh soil (5 g) was suspended in 50 ml of extracting solution [0.5 M trichloroacetic acid (TCA) and 0.25 M Na_2PO_4]. After stirring, soil samples were sonicated for 3 min., and the suspensions were centrifuged at 1500 g for 5 min at 4 °C. Aliquots of 50 μl were taken from the supernatant and were transferred into polystyrene tubes, mixed with 4.95 ml of EDTA-Tris acetate buffer (0.1 M Tris, 2 mM EDTA), pH 7.75, and vortexed for 10 s. Aliquots of 150 μl were tested for ATP by adding them to a buffered luciferin–luciferase solution.

The radiation emission of the mixtures was measured with a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA, USA). The estimate of the soil ATP content was done in triplicate. Autoclaved soil extracts were used to obtain blank values. Counts over a 10 s integration time were compared with a standard curve of ATP.

2.7. *Experimental design and statistics*

This study was designed as a randomized complete block, multi-factorial, double-nested with sub-sampling experiment.

Table 5 – Effects of *Bt* maize on dehydrogenase activity (mean μg TPF·g^{-1} 24 h^{-1} dry soil)

	Elgina (t)	Cecilia	Compa (t)	Dracma	Average
Sowing	13.45	15.85	19.55	16.70	**16.39**
Rhizosphere	16.10	17.60	22.50	20.20	19.10
Non-rhizosphere	10.80	14.10	16.60	13.20	13.68
Harvest	43.40	38.70	27.80	28.05	**34.49**
Rhizosphere	46.60	48.00	33.20	36.10	40.98
Non-rhizosphere	40.20	29.40	22.40	20.00	28.00
Average	**28.43**	**27.28**	**23.68**	**22.38**	25.44
Tukey's HSD	a	a	b	b	

(t) = transgenic line. See Table 2 for explanation.

The block treatment was the two years of sampling and the four maize lines were the main source of variation. The interaction of blocks and maize lines was used for significance testing of the blocks and maize lines. Nested within these were the two sampling dates and the rhizosphere/non-rhizosphere soils, each with two levels. These treatments were tested with their interaction mean squares, and the latter with the residual error from the sub-sampling. These analyses of variance (ANOVAs) were performed in two steps: the calculations of the mean squares were done first with *JMP* 6, and the *F* tests then calculated by hand. Because the differences among the maize lines were the principal subject of interest, the means were subsequently tested *a posteriori* to the ANOVAs using Tukey's HSD mean separation test at the 0.05 probability level.

3. Results

Six ANOVAs were calculated to test for significant differences among the treatment means. Given the complexity of the ANOVA model, an example is given in Table 1 for the bacteria data. The remaining five ANOVAs are not presented. Maize line was not a significant source of variation in any of the six ANOVAs. Year, which is the block term, was significant in four of the six ANOVAs, which then resulted in the year by maize line interaction source of variation being significant for all six variables. Therefore the interaction term was used to test the maize line source of variation. It is the only variation at the same scale that can be used in the *F* test. In general, values for the different microbiological parameters under analysis were higher in Year 2, which were likely related to differences in weather conditions.

Sampling date was significant in three of the ANOVAs, while rhizosphere/non-rhizosphere soils were significant in only two ANOVAs.

The numbers of culturable heterotrophic aerobic bacteria (Table 2) and actinomycetes (Table 3) showed no statistically or biologically significant differences between soils planted with *Bt* or non-*Bt* maize. Some significant differences were observed in the abundance of saprophytic fungi (Table 4). Fungal populations were lower 30 days after sowing in soils with Compa Cb transgenic maize when compared with its isogenic line Dracma. Maize lines did have statistically different fungal population means, but they were not biologically meaningful. This effect did not persist at pre-harvest date, where values did not show significant differences. Between sampling dates numbers of these three major soil microbial groups were higher at pre-harvest date. Large number of heterotrophic bacteria was also detected in the rhizosphere soil. In contrast, actinomycetes and fungi were not, in general, stimulated in the rhizosphere.

The variations in soil DHA activity and ATP content are shown in Tables 5 and 6, respectively. The presence of *Bt* maize did not affect, in a general way, these soil parameters. The values in rhizosphere soil were higher, in general agreement with the microbial counts.

A marked influence of the presence of maize roots was detected in the nitrogenase activity (Table 7), as values were significantly higher at the pre-harvest date in the rhizosphere soil. However, the presence of transgenic maize lines did not significantly affect nitrogenase activity.

In spite of some individual cases of reduced populations under *Bt* compared with non-*Bt* maize, these reductions were transient and did not persist between sampling times.

Table 6 – Effects of *Bt* maize on soil ATP content (mean ng·g^{-1} dry soil)

	Elgina (t)	Cecilia	Compa (t)	Dracma	Average
Sowing	270.70	239.25	246.25	281.75	**259.49**
Rhizosphere	298.90	285.50	287.50	360.00	**307.98**
Non-rhizosphere	242.50	193.00	205.00	203.50	**211.00**
Harvest	357.00	427.50	467.50	421.50	**418.38**
Rhizosphere	470.50	544.00	565.00	514.00	**523.38**
Non-rhizosphere	243.50	311.00	370.00	329.00	**313.38**
Average	**313.85**	**333.38**	**356.88**	**351.63**	338.93
Tukey's HSD	b	ab	a	a	

(t) = transgenic line. See Table 2 for explanation.

Table 7 – Effects of *Bt* maize on nitrogenase activity (mean nmoles $C_2H_4 \cdot g^{-1} \cdot 24\ h^{-1}$ dry soil)

	Elgina (t)	Cecilia	Compa (t)	Dracma	Average
Sowing	11.75	9.95	12.25	13.95	11.98
Rhizosphere	18.40	14.10	19.40	22.00	**18.48**
Non-rhizosphere	5.10	5.80	5.10	5.90	**5.48**
Harvest	89.85	95.00	235.75	208.75	157.34
Rhizosphere	111.90	168.70	422.80	379.00	**270.60**
Non-rhizosphere	67.80	21.30	48.70	38.50	**44.08**
Average	**50.80**	**52.48**	**124.00**	**111.35**	84.66
Tukey's HSD	b	b	a	a	

(t) = transgenic line. See Table 2 for explanation.

4. Discussion

We evaluated the effects of *Bt* maize under typical field conditions, within large plots. The results indicated that *Bt* maize did not cause significant effects on the variables measured. Occasional significant differences did not persist. Our field-based results are consistent with other experiments (Donegan et al., 1995; Saxena and Stotzky 2001; Devare et al., 2007) indicating that the transgenic corn had no discernable effect on the bacterial community. In a microcosm experiment with *Bt* transgenic cotton leaves, Donegan et al. (1995) observed that two of the three transgenic lines produced a significant but transient increase in the number of CFUs of culturable bacteria and fungi, and Saxena and Stotzky (2001) found that there were no significant differences in the CFUs of culturable bacteria, actinomycetes or fungi between soil amended with biomass of *Bt*-corn and non-*Bt*-corn after 45 days of incubation. Studies conducted by Wu et al (2004) on Bt rice (Cry1A) in China suggested that *Bt* rice had no

negative effect on a range of soil microbial indicators. In contrast, some antagonistic effects of the Cry insecticidal protein on ammonification and nitrification have been reported (Visser et al., 1994), but other studies have shown no deleterious effects (Casida, 1989). Glare and O'Callaghan (2000) reported that many of the effects on microorganisms were short term and concluded that it was unlikely that there would be any lasting effects on soil microbial processes. In another study on *Bt* proteins, an experiment carried out under laboratory conditions to investigate the differences in the population of culturable microorganisms and the enzymatic activities between soils amended with straw of transgenic rice containing the Cry1Ab gene and the isogenic non-transgenic rice showed no significant differences in the CFU of culturable bacteria, actinomycetes and fungi between the two soils (Weixiang et al., 2004).

We did observe some stimulatory rhizosphere effects on measured variables as compared to non-rhizosphere soil. These findings are in keeping with previous work demonstrating that increases in plant root exudation result in increased microbial activity (Swinnen, 1994; Ryan et al., 2001).

Among the many essential functions of the soil biota are microbially-mediated processes related to nutrient cycling, such as oxidation–reduction reactions and biological N_2-fixation (Motavalli et al., 2004), but relatively little research has examined the effects of transgenic crops on these processes and functions in soil (O'Callaghan and Glare, 2001; Bruinsma et al., 2003). In this study some differences were observed in nitrogenase activity, namely between rhizosphere soil of Compa Cb and its near-isogenic line (Dracma), in year 1. N_2-fixing activity also seemed to be more sensitive to changes in soil conditions, as ARA showed a marked increase in the rhizosphere when compared with the other microbiological parameters measured. There were clear seasonal effects on microbial biomass and activity in our field plots, as represented by the consistent changes in all measured variables across years and sampling dates. In year 1, most of microbiological parameters evaluated showed a severe reduction probably due to unusually dry season.

Because dehydrogenases are not active as extracellular enzymes in soil, independent of the parent microbial cell, the measurement of DHA is a good overall indicator of microbial activity. DHA has been used as an indicator of the microbiological activity in semi-arid Mediterranean soils (Garcia et al., 1994), in agricultural soils in Germany (Beyer et al., 1982), in Mediterranean soils during the dry and wet seasons (Quilchano and Marañón, 2002), and in soil management studies (Bergstrom et al., 1998). Differences in DHA could also be the result of differences in the composition of microbiota in stressed soil (Leirós et al., 2000). ATP is the most important and central coupling agent between exergonic and endergonic processes in all cells; in dead cells ATP is quickly degraded. Owing to its properties, ATP is proposed as a parameter for either estimating microbial activities or biomass in soil. Soil ATP is also closely related with other indices of biomass, e. g. C, N, etc. and can serve as an independent estimate of soil biomass content (Contin et al., 2001).

In the present study, an evaluation of DHA and ATP content in soils under *Bt* and non-*Bt* maize was undertaken. The values obtained for both soils (rhizosphere and non-rhizosphere) showed no significant differences.

Among the potential direct effects are changes in soil microbial activity due to differences in the amount and composition of root exudates. The insecticidal protein in root exudates binds rapidly to clay minerals and humic substances, which protect the protein from microbial degradation (Tapp et al., 1994; Crecchio and Stotzky, 1998; Saxena et al., 2002). The bound protein retains its insecticidal activity and has been observed to persist in soil up to 234 days (Saxena et al., 1999). Similar results on the persistence of the insecticidal protein of *Bt* cotton, but for shorter periods of time (up to 140 days), have also been reported (Palm et al., 1996). However, in a survey of the levels of Cry1Ac protein in soil samples of six fields with continuous *Bt* cotton for 3 to 6 years, Head et al. (2002) indicated that no detectable Cry1Ac protein or biological insecticidal activity was present in any of the fields. Tapp and Stotzky (1998) found that insecticidal activity was retained when the Cry protein was incubated in soil, but the amount of retention varied with the type of soil, probably because of differences in the clay mineral composition and the pH of the soils. Since clay content increases the retention of Cry proteins (Crecchio and Stotzky, 1998), the protein probably does not persist for a long period in our soil conditions since the soil under study was sandy and well drained. Although the Cry protein level has not been evaluated we can presume that if the Cry proteins do not bind on clays they are available to soil microbes and a short term effect on the microbial parameters under study could be expected, but such an effect was not observed for the first sampling date.

Our study over two years in the same plots suggested there were no cumulative effects of *Bt* maize, at least in the short term. The differences caused by growing *Bt* maize were not as large as those resulting from rhizosphere and seasonal changes indicating that the effect of *Bt* maize on soil microorganisms was within the normal variation expected in conventional agricultural systems.

Acknowledgement

This research was supported by Project AGRO 17, under AGRO Program — Medida 8 — Acção 8.1.

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