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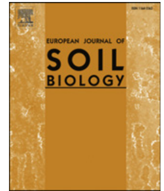
European Journal of Soil Biology xxx (2009) 1–8

Contents lists available at ScienceDirect

European Journal of Soil Biology

journal homepage: http://www.elsevier.com/locate/ejsobi



Original article

Release of the recombinant Cry3Bb1 protein of *Bt* maize MON88017 into field soil and detection of effects on the diversity of rhizosphere bacteria

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A R T I C L E I N F O

Article history:
Received 24 August 2009
Received in revised form
27 October 2009
Accepted 27 October 2009
Available online xxx
Handling editor: Bryan Griffiths

Keywords:
Genetically engineered maize
Bt maize MON88017
Cry3Bb1 protein
Bacterial community analysis
PCR-amplified 16S rRNA genes
Rhizosphere soil

A B S T R A C T

A three-year experimental field study with a genetically engineered *Bt* maize (event MON88017) and three conventionally bred cultivars was conducted to quantify the recombinant Cry3Bb1 protein released into soil and detect effects on the diversity of soil bacteria. Protein extraction and an enzyme-linked immunosorbent assay (ELISA) allowed a threshold detection of 0.01 ng Cry3Bb1 g^{-1} soil. The maximum amount found in field plots with *Bt* maize was 1.0 ng Cry3Bb1 g^{-1} rhizosphere soil. Average concentrations during the growing seasons varied between years from 0.07 to 0.29 ng g^{-1}. No accumulation of Cry3Bb1 in soil occurred over the three growing seasons. Four weeks after harvest, the major Cry3Bb1 reservoirs on the field were the remaining root stubbles, but their Cry3Bb1 concentration declined by 98.30–99.99% in the following seven months. During the three consecutive years of study there were never significant differences between the rhizosphere bacterial community structure of the *Bt* maize and the other cultivars, as detected by cultivation independent profiling of PCR-amplified 16S rRNA genes. The low concentrations of soil extractable Cry3Bb1, its degradation in decaying roots, and the lack of effects on rhizosphere bacteria give no indications of adverse effects of MON88017 cultivation on soil ecology.

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1. Introduction

In the year 2008, genetically engineered plants were cultivated worldwide on an estimated total area of 125 million hectares [24]. A significant portion of these plants were *Bt* crops, which were modified to express insecticidal Cry-proteins (*Bt*-toxins, *Bt*-proteins). In nature, Cry-proteins are delta-endotoxins produced by various bacterial strains, subsumed under the name *Bacillus thuringiensis* [7]. Due to their specificity towards certain target pest organisms, *Bt*-proteins can be used for biocontrol [12,18] and, thus, they are potentially attractive alternatives to synthetic chemical insecticides. *Bt*-proteins can be used directly as formulated products or they can be synthesized by crops once the property has been introduced and expressed by genetic engineering [14,18].

Before introduction to agriculture, new *Bt* crops should be evaluated for their effects on non-target soil organisms and ecology [1,38]. Despite the experience already gained by their worldwide cultivation, the diversity of now available *Bt*-toxins, the different genetic modifications of the plants at the molecular level, and the specific environmental conditions existing in the chosen agricultural ecosystems are all important variables that deserve a case-by-case analysis for environmental risk assessment [13,45]. One of the key issues in this context is the question of how much recombinant product, in this case the Cry-protein, is released into agricultural field soils by a crop, how it may interact with the abiotic and biotic soil components and how long it will persist after crop harvest.

The objective of this study was to quantify the amount of Cry3Bb1 proteins released from *Bt* maize DKC5143Bt (event MON88017) into agricultural soil, to follow its fate over a period of three years and to analyze whether cultivation of this crop modifies the bacterial diversity in soil to an extent beyond that known from non-engineered plants. MON88017 provides resistance against the Western Corn Rootworm *Diabrotica virgifera*, an agricultural pest of maize with obviously increasing importance in Europe [10,20]. An experimental field site was set up in the central region of Germany. It included eight replicated field plots for each, the *Bt* maize and three non-modified maize varieties. In order to consider cumulative effects, cultivations of the specific varieties were conducted each year on the same plots. Cry3Bb1 proteins were detected by an enzyme-linked immunosorbent assay (ELISA) using a modified protocol with a sensitivity of detection not reported before. Potential changes of the soil bacterial diversity living in the immediate vicinity of the maize roots, i.e., the rhizosphere, were analyzed by cultivation-independent

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doi:10.1016/j.ejsobi.2009.10.003

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genetic fingerprinting of the universal bacterial 16S rRNA genes directly extracted from soil DNA [43]. This allowed the detection and comparison of the major members of the bacterial communities independent of their capacity to be cultivable under laboratory conditions.

2. Materials and methods

2.1. Maize cultivars, field site and sampling

The *Bt* maize variety under investigation was the hybrid DKC5143Bt (event MON88017; Monsanto, St. Louis, Mo) which expresses the insecticidal *Bt*-toxin Cry3Bb1 due to the cry3Bb1 gene of *B. thuringiensis* var. *kumamotoensis* and the CP4 EPSPS protein, encoded by the cp4 *epsps* gene of *Agrobacterium* sp. strain CP4, the latter conferring resistance to glyphosate [21]. By production of Cry3Bb1, the hybrid becomes resistant against the Western corn rootworm (*D. virgifera virgifera*), a member of the leaf beetle family (Coleoptera: Chrysomelidae).

A report by the Canadian Food Inspection Agency (2006) (http://www.inspection.gc.ca) on MON88017 indicates expression levels of 100–370 μg g^{-1} dry weight (dw) root tissues. Non-*Bt* maize varieties used in this study as controls (comparators) included the near isogenic counter part DKC5143 as well as the varieties DK315 (Monsanto) and Benicia (Pioneer Hi-Bred, Johnston, IA).

The site of this study was a 4-ha agricultural field near Würzburg (Bavaria, Germany). It was characterized by heterogeneity of three different soil types, i.e., a cambisol, a stagnic luvisol, and mazi-eutric vertisol (personal communication, S. Pagel-Wieder, 2008). The experimental field included the 32 plots analyzed in this study with each of four different maize varieties in 8 replicates, ordered in a block randomized field design consisting of four rows (A to D). Each plot covered an area of 31.5 m by 40.5 m. The field site was surrounded by a 10 m clearance strip and a 10 m wide perimeter of the conventional maize variety Gavott (KWS AG, Einbeck, Germany). The field trial was performed from 2005 to 2007, including three growing seasons with maize cultivations.

Cry3Bb1 concentrations were analyzed in both bulk soil samples and soils from maize rhizospheres. During the vegetative period, samples were taken according to specific growth stages of the maize plants, i.e., BBCH 20 (nine leaves per plant), BBCH 30 (stem elongation phase), BBCH 60 (flowering, anthesis), and BBCH 80 (ripening) [27]. In addition, bulk soil samples and root stubbles were collected 4 weeks after harvest and at the beginning of May, directly before maize seeding for the following season.

Bulk soil was collected from each plot at five randomly selected points between plant rows. Rhizosphere soil was collected from 3 to 8 individual plants depending on their growth stage. Plants were carefully dug out to prevent root damage. The soil adhering to the roots was then collected in plastic bags. In the laboratory, plant and root residues were removed from the soil samples with forceps, followed by sieving (mesh size, 2 mm). The individual samples from each field plot were combined and stored at −20 °C.

Soil for bacterial community analyses was obtained after transporting plants with roots and adhering soil as well as root stubbles to the laboratory where they were further processed for DNA extraction (see below).

2.2. Extraction and quantification of Cry3Bb1 protein

The Cry3Bb1 protein (*Bt*-toxin) in all materials was determined with a double antibody sandwich ELISA using a commercially available test system (PathoScreen™ kit for *Bt*-Cry3Bb1 protein; Agdia, Elkhart, Indiana). The roots were briefly rinsed with sterile distilled water to remove soil particles, and 5 g of this material (fresh weight equivalent) was ground in a mortar with liquid nitrogen. The crushed plant tissue was then stored at −70 °C until further processing. Aliquots of approximately 50 mg of crushed plant tissue samples were placed into 1.5-ml micro-reaction tubes (Eppendorf, Hamburg, Germany) and mixed for 30 s with 0.5 ml extraction/dilution buffer (supplied with the kit) using plastic pestles. The resulting suspensions were centrifuged, and the clarified supernatants were analyzed by ELISA, following the recommended protocol of the manufacturer.

To extract the Cry3Bb1 protein from soil, three parallels of 1 g wet weight soil of each sample were separately mixed each with 3 ml of the extraction/dilution buffer and homogenized on a VF2-vortex (Janke-Kunkel IKA-Labortechnik, Staufen, Germany) at maximum speed. The soil suspensions were centrifuged for 30 min at 16,000 × *g* and 4 °C. The three parallels were combined and concentrated five-fold by ultrafiltration with Microcon YM-10 centrifugal filter devices (Millipore, Eschborn, Germany). The sensitivity of the ELISA was increased by extending the recommended 1-h incubation period of the Cry3Bb1-enzyme-conjugate to 17 h at 4 °C in the dark. For quantification of the Cry-protein, positive controls supplied by the manufacturer were diluted with extraction/dilution buffer to reach final concentrations of 0.1, 0.2, 0.4, 0.8, 1.25, 2, and 2.5 ng Cry3Bb1 ml^{-1}, respectively. These dilutions were used to set up calibration curves. The ELISA quantification method has previously been evaluated [35]. Significances among data were determined by the one-way analysis of variance (ANOVA) and means were compared with the Tukey-test (all pairwise multiple comparisons) using the SigmaStat (V.2.03) program package (SPSS, Chicago, Illinois).

2.3. Bacterial community analysis by single strand conformation polymorphism (SSCP) of PCR-amplified 16S rRNA genes

Rhizosphere bacteria were collected from maize plants (BBCH 60) and their total DNA was extracted as described previously [4]. Briefly, bacterial cell pellets collected by centrifugation of saline extracted root suspensions were extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, Heidelberg, Germany) according to the recommended protocol using the bead beating in a FastPrep-Instrument (model FP120; Qbiogene, Irvine, Ca) for cell lysis.

For total bacterial community analysis, ribosomal RNA genes of the purified DNA solutions were amplified using the universal primers Com1 and Com2Ph [16,44]. Selected phylogenetic groups of the bacterial community were amplified with taxon-specific primers followed by a second PCR (nested PCR) with the universal Com-primers mentioned above. Primers used to amplify members of *Alphaproteobacteria* were F203alpha and R1492 [19,48] and for *Pseudomonas* F311Ps and R1459Ps [33]. The PCR amplifications for each sample were carried out in three parallels and the resulting products were combined, analyzed for size and quantity by agarose gel electrophoresis, and purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) [4,16].

Approximately 700 ng of purified PCR product were incubated at 37 °C for 45 min with 5 U of lambda-exonuclease (New England BioLabs, Frankfurt, Germany) to remove the phosphorylated reverse DNA strands. The single-stranded DNA was then purified with the MiniElute PCR purification kit (Qiagen) and loaded on non-denaturing, temperature controlled, vertical polyacrylamide gels for SSCP electrophoresis and separated for 17 h at 20 °C and 400 V in a Macrophor apparatus [16,47]. After the electrophoresis, the DNA was visualized by silver staining [3]. Statistical analyses were only conducted with SSCP profiles from gels which had been loaded with the respective rhizosphere samples in a randomized order.

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2.4. Digital image analysis of SSCP profiles

SSCP profiles were digitalized using a flatbed scanner with a light source in the lid (ScanJet 4C/T, Hewlett Packard, Böblingen, Germany). The images were then converted into densitometric values and analyzed with the GelCompar program package II (version 4.5; Applied Math, Kortrijk, Belgium). Individual SSCP patterns were normalized by using a migration marker [41]. Calculation of the similarity matrix was based on Pearson correlation coefficients. UPGMA (unweighted pair group method using arithmetic averages) was applied for clustering profiles based on their similarity. The significance of clusters was analyzed by permutation tests based on the similarity matrices [26].

3. Results

3.1. Quantification of Cry3Bb1 protein in rhizosphere and bulk soil

Immunoreactive Cry3Bb1 proteins were quantified in soil from field plots with MON88017 over a period of three consecutive growing seasons. For each sampling, soil was taken from all eight replicate plots and, in addition, from two plots with the near-isogenic

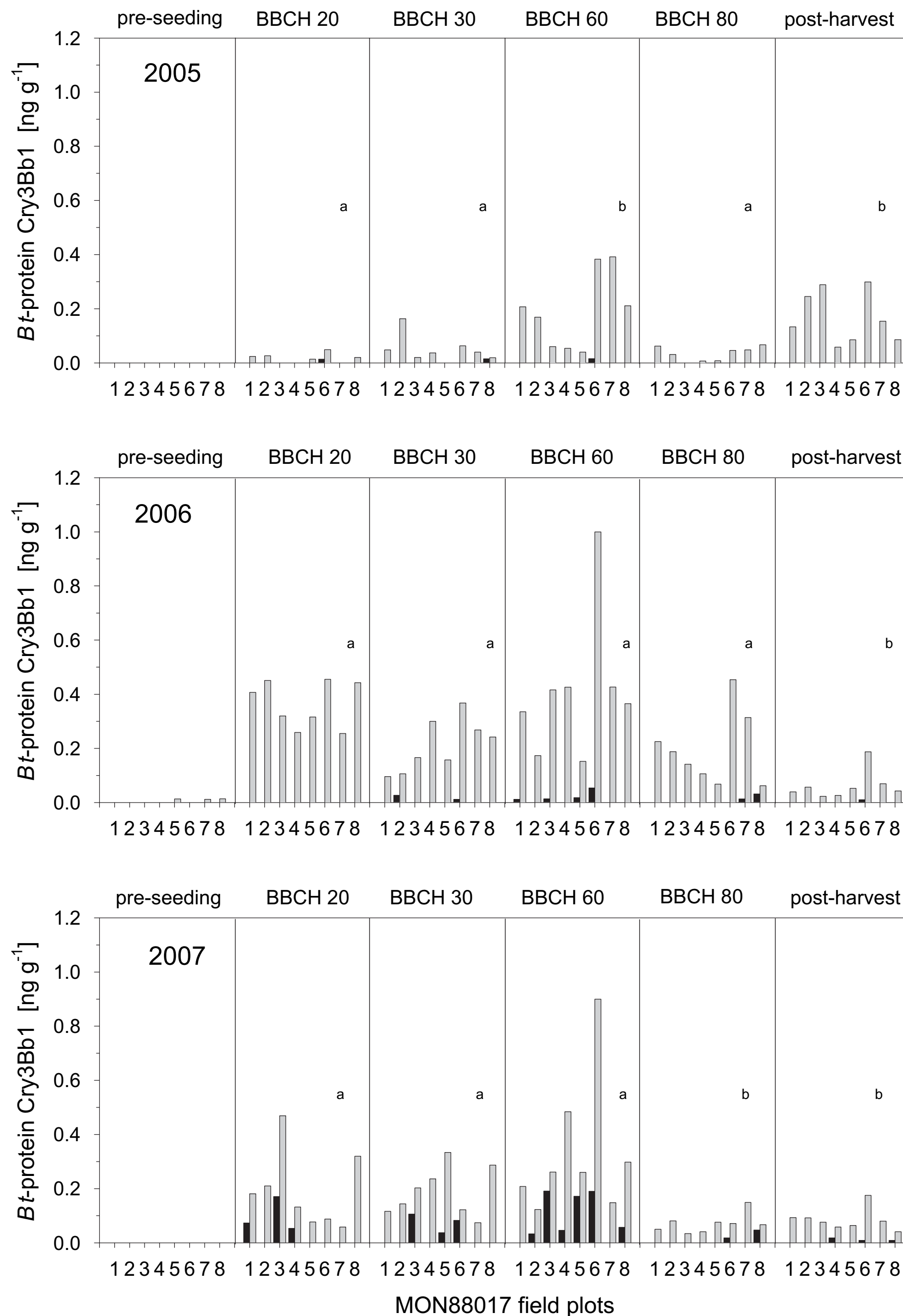

Fig. 1. Quantification of the Cry3Bb1 protein in bulk (black bars) and rhizosphere soil (grey bars) on an experimental field site cultivated with the *Bt* maize MON88017 throughout three consecutive growing seasons (years 2005–2007). Numbers at the *x*-axis indicate the replicate field plots analyzed separately from each other. Growth stages of maize at the time of sampling are indicated by BBCH 20 (nine leaves per plant), BBCH 30 (stem elongation phase), BBCH 60 (flowering, anthesis), and BBCH 80 (ripening). "Post-harvest" indicates sampling one month after harvesting. Growth stages marked with the same letters were not significantly different ($P < 0.05$) in respect to their mean value of Cry3Bb1 in rhizosphere soil (separate analyses for each year).

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non-transgenic cultivar DKC5143. The Cry3Bb1 protein was never detected in samples taken from the non-transgenic plots while the Cry3Bb1 protein was detected in rhizosphere and, infrequently, also in bulk soil samples from the MON88017 cultivated plots (Fig. 1).

In rhizosphere soil, the Cry3Bb1 was detected for the different samplings in concentrations ranging from 0.01 to 1.0 ng g^{-1} soil or its detection was negative (<0.01 ng). The average concentration of Cry3Bb1 during the growing season (BBCH 20–BBCH 80) for the first cultivation period (year 2005) was 0.07 ng g^{-1}, while it was 0.29 ng g^{-1} for the second year of cultivation and 0.19 ng g^{-1} soil for the third (2007). The overall mean value for three years for the maize cultivation period was 0.18 $\pm$ 0.1 ng g^{-1} rhizosphere soil (Fig. 1). In general, the concentrations of Cry3Bb1 found at the different growth stages were not significantly different, except for BBCH 60 in the first year with significantly elevated concentrations and for BBCH 80 in the third year with significantly lower values. The highest concentrations were always detected at BBCH 60, but this was only significant in 2005 ($P < 0.05$). After harvest, the amount of Cry3Bb1 in the soil adhering to the remaining root stubbles, the former rhizosphere, decreased significantly in both years analyzed, i.e., for 2006 and 2007, respectively. Seven months after harvesting, the concentration of the Cry3Bb1 protein had declined to values around the threshold of detection on 3 single plots in 2006 or below (all other samples in 2006, all samples in 2007) (Fig. 1).

In bulk soil, Cry3Bb1 mean concentrations were significantly lower than those measured in the corresponding rhizosphere soil (Fig. 1) ($P < 0.05$; for all sampling dates). In the first season, the protein could be detected only on three occasions throughout the growing season. Concentrations were as low as 0.014–0.016 ng g^{-1} soil. In the second and third year the frequency of detection increased. The maximum concentration detected, was 0.19 ng g^{-1} soil on two replicate plots at the growth stage BBCH 60 in the third year. These values paralleled the elevated levels of Cry3Bb1 found in the corresponding rhizosphere soil. The annual average concentration for Cry3Bb1 in bulk soil in 2007 was 0.034 $\pm$ 0.03 ng g^{-1}, which was 5-fold lower than the average concentrations of the rhizosphere soil.

3.2. Quantification of the Cry3Bb1 proteins in root residues of maize stubbles

Roots of plant stubbles remaining on the field were collected between three and four weeks after the maize plants had been harvested. Furthermore, another sampling of the same material was conducted directly before seeding of maize in early May of the following year. In October 2005 and 2006, the analysis of these root residues resulted in average concentrations of 1069 $\pm$ 408 and 336 $\pm$ 171 ng of immunoreactive Cry3Bb1 protein g^{-1} fw, respectively (Fig. 2). This corresponded to approx. 15% and 5% of an average concentration of Cry3Bb1 found in roots of MON88017 sampled from the same field plots in a parallel study shortly before harvesting, at BBCH83 [34]. In samples collected 7 months later (May 2006), Cry3Bb1 levels from residues of maize plants cultivated in 2005 decreased to 0.15 ng g^{-1} on average, corresponding to a decline by 99.99%. During the season, the Cry3Bb1 levels further declined below the limit of detection with an exception of four single occasions with maximum value of 0.6 ng Cry3Bb1 g^{-1} root material (fw). Despite the fact that the Cry3Bb1 concentration in root stubbles of plants grown in 2006 was lower in October, compared to the previous season, the Cry3Bb1 concentration in May (year 2007) was slightly higher with an average of 5.7 ng

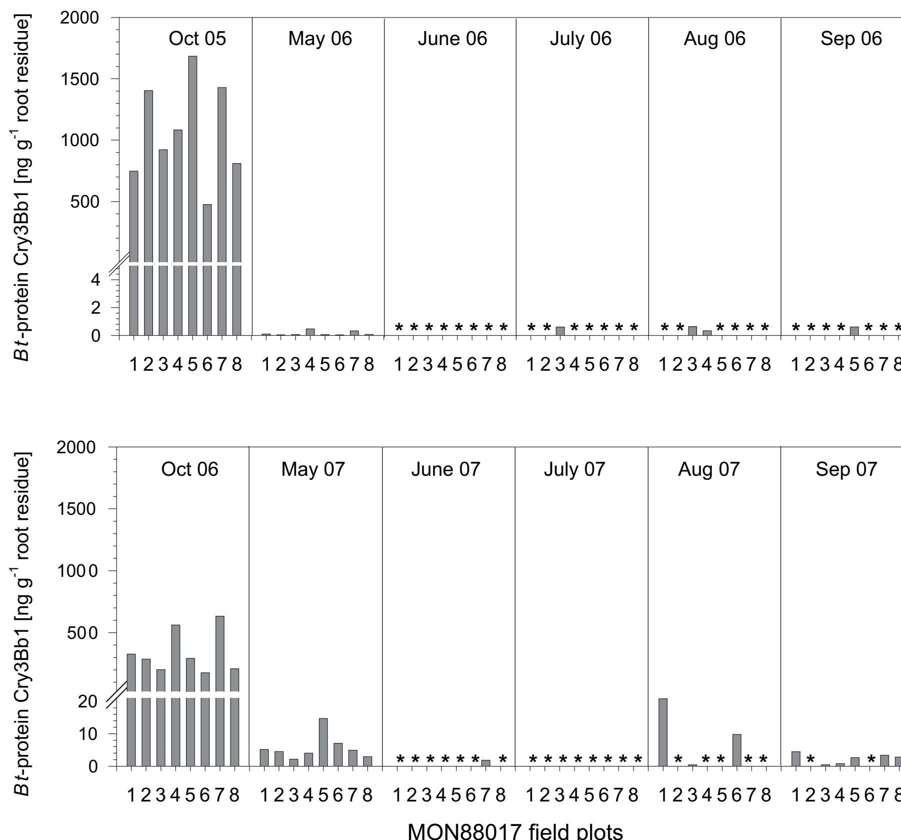

Fig. 2. Persistence of Cry3Bb1 protein in decomposing plant residues after harvest in 2005 and 2006. All values refer to fresh tissue weight. *Indicates "below detection limit".

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Cry3Bb1 g^{-1} root material, thus, seeing a decline of 98.30% during the winter season. Furthermore, Cry3Bb1 protein in the decomposing root stubbles from 2006 was detected more frequently in the following months with concentrations up to 20 ng g^{-1}.

Differences in Cry3Bb1 degradation detected between the two winter seasons was accompanied by different weather conditions: The winter 2005/2006 (October–May) was dry and cold with average temperatures of 3.7 °C and a total of 61 frost days, while the winter 2006/2007 was characterized by mild temperatures (average 7.4 °C), higher rainfall (306 mm, compared to 239 mm in 2005/2006), and only 10 frost days.

3.3. Bacterial diversity in the rhizosphere of MON88017 and other maize cultivars

The bacterial diversity found in the rhizosphere soil of MON88017 was analyzed from plants collected at growth stage BBCH 60, as these tended to have the highest Cry3Bb1 concentrations (see Fig. 1). Conventionally bred cultivars of the same growth stage with no potential to produce Cry3Bb1 were included.

SSCP profiles targeting all dominant bacteria were composed of approximately 30–40 distinguishable bands (Fig. 3A). SSCP profiles targeting *Alphaproteobacteria* were composed of a similar number of bands, but profiles for the *Pseudomonas* group showed only approx. 15 bands (Fig. 3B and C). The specificity of the applied primers for SSCP analyses of maize rhizosphere for the targeted groups had been established in a previous study [6]. It should be noted that the following similarity analyses were conducted with gels loaded in a randomized order while Fig. 3 shows gels loaded according to the field rows from which the rhizosphere samples were obtained. High similarities, mostly in the range of 80% and above, were seen between all profiles for each of the targeted bacterial groups, independent of their cultivar or the vicinity of field plots from which they originated (Fig. 4). Unstable clustering of individual field plots occurred with SSCP profiles of the same bacterial groups from different years, suggesting that the environmental conditions like weather or annual, non-characterized field heterogeneities were responsible for such minor differences. Permutation tests using the similarity matrices of the cluster analyses were applied to establish the significance of differences between MON88017 and the non-engineered cultivars. P values were always above the significance level ($P > 0.05$), underlining the lack of effects of the genetically engineered variety on the diversity of the most abundant *Bacteria*, *Alphaproteobacteria* and members of the *Pseudomonas* group.

4. Discussion

Cry3Bb1 proteins were detected in this study on all sampling dates throughout three years of sampling in rhizosphere soil of the *Bt* maize. No Cry3Bb1 was detected in rhizospheres of the non-engineered near isogenic and other cultivars growing on the same field, which demonstrated the specificity of the applied ELISA assay for soil analyses. Two previous field studies quantified the amount of Cry3Bb1 released from *Bt* maize and reported about a complete lack of its detection in soil during the growing season, even when the same cultivar had been cultivated for three or four consecutive years in the same site [2,23]. In our study the event MON88017 was analyzed while in the other two studies MON863 was examined, but expression levels of both events are similar, mainly in a range between 100 and 200 μg Cry3Bb1 per g tissue dw [31,34,36]. However, protein extraction from soil in this study was followed by a five-fold protein concentration step and incubation during the detection of the fluorescent product in the ELISA was extended. These two modifications allowed the threshold to be optimized to 0.01 ng Cry3Bb1 g^{-1} soil in comparison to previously reported

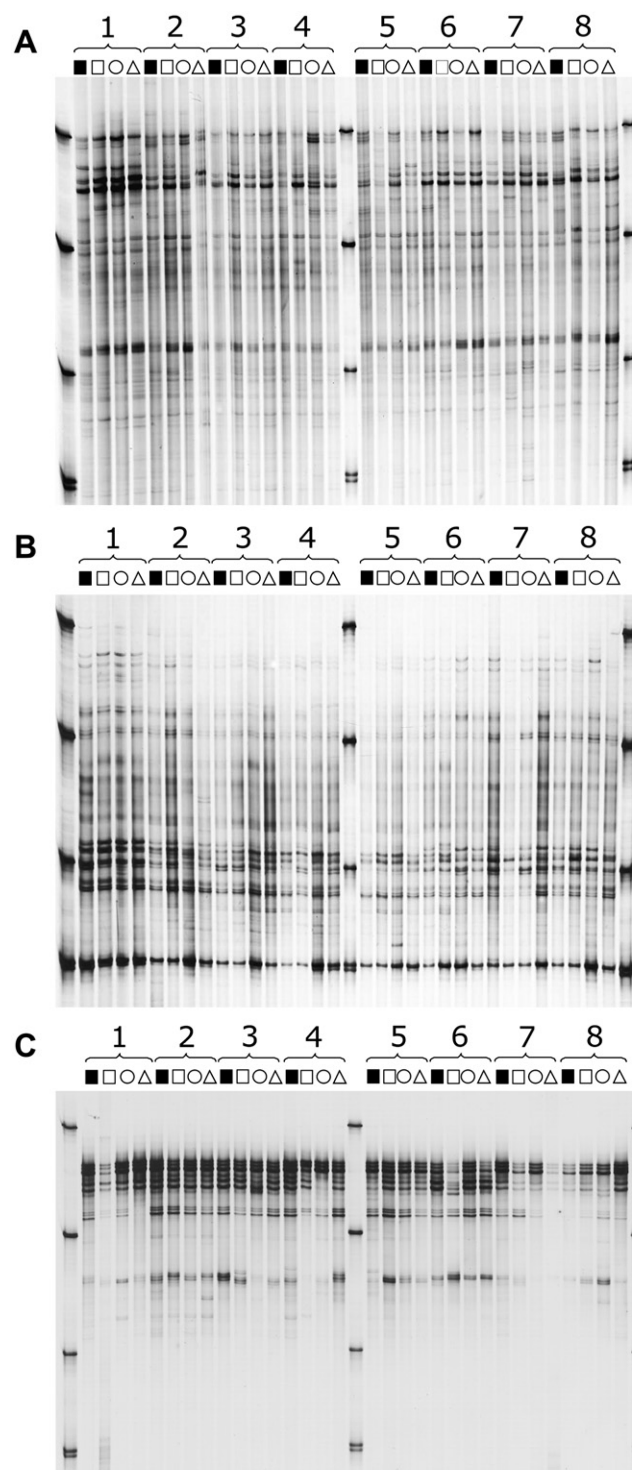

Fig. 3. SSCP profiles of the rhizosphere inhabiting bacterial communities from maize cultivars at growth stage BBCH 60 (samples from the year 2006). Partial 16S rRNA genes were PCR-amplified from directly extracted soil DNA. SSCP profiles targeting the domain *Bacteria* (A.), the subclass *Alphaproteobacteria* (B.), and the *Pseudomonas* group (C.), the latter two using nested PCR and specific PCR-primers. Outer lanes of SSCP gels show SSCP markers. Numbers above each gel indicate the row of the plots in the field, symbols the different maize varieties, i.e., ■ for MON88017, □ for DKC5143 (near isogenic line), ○ for Benicia, and Δ for DK315, respectively.

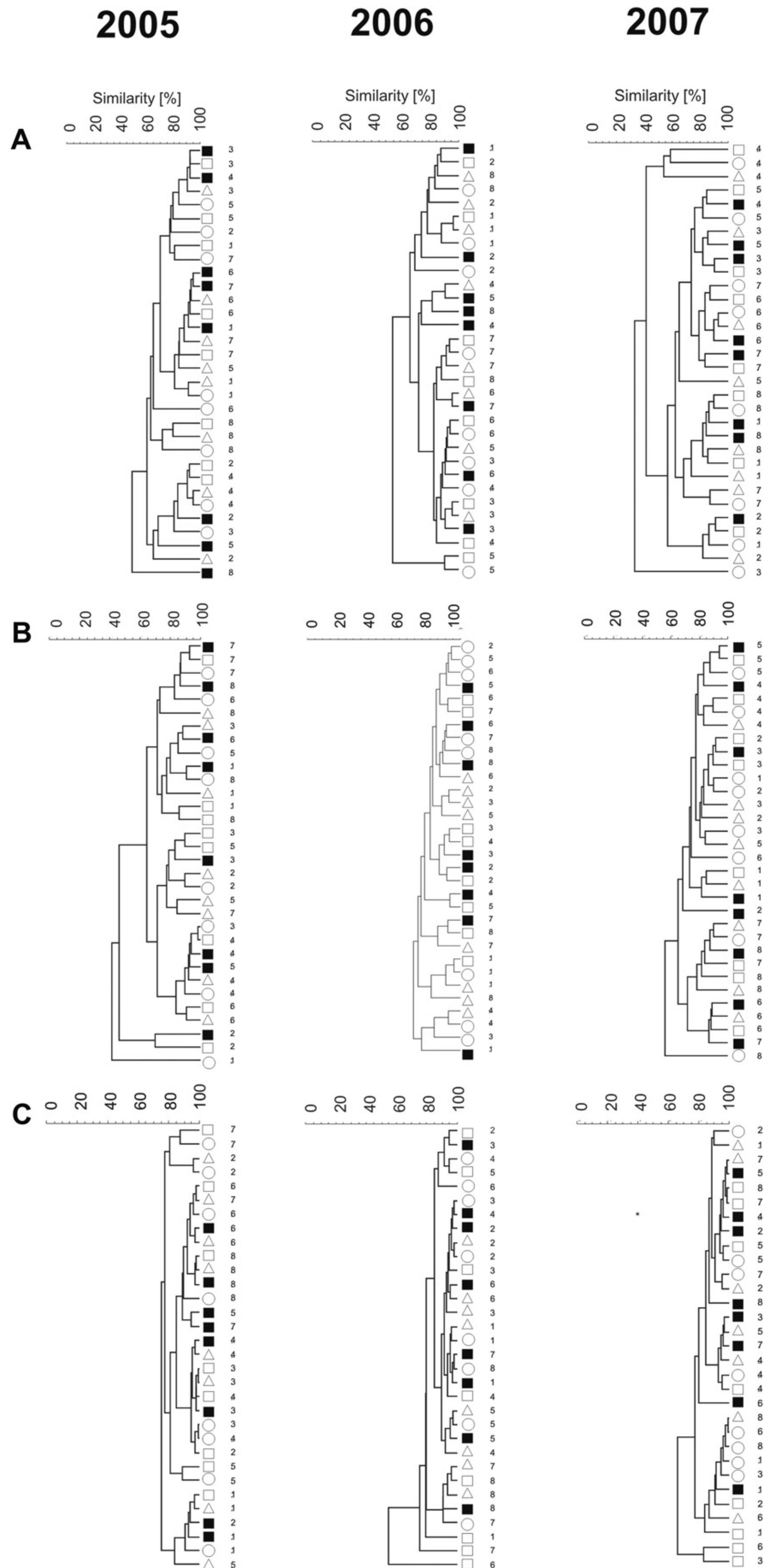

Fig. 4. Cluster analyses of bacterial communities of maize rhizospheres (growth stage BBCH 60) collected during three consecutive growing seasons (2005–2007) as detected by PCR–SSCP analysis using primers targeting *Bacteria* (A.), *Alphaproteobacteria* (B.) and members of the *Pseudomonas* group (C.). Similarity matrices based on Pearson correlation and UPGMA (unweighted pair group method with arithmetic averages). Note that genetic profiles were generated from SSCP gels with samples in a randomized order to avoid analytical similarities due to gel-based heterogeneities. For symbols of maize varieties, see legend of Fig. 3. Numbers next to symbols indicate the field row of the plot from which maize rhizosphere samples were obtained.

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0.7 ng g^{-1} soil [2,36]. Other factors which explain differences of Cry3Bb1 detection to the previous field studies may relate to properties of the extraction buffer (pH, ionic strength) and composition of the soils (pH, clay content).

A lack of detection of Cry3Bb1 indicated by ELISA techniques in this or in other studies does not necessarily mean that the protein is actually absent. Extraction of Cry3Bb1 from soil using only water allowed recovery of less than 0.1% of 36 μg Cry3Bb1 added [31]. This suggests strong adsorption of Cry3Bb1 to surface active soil particles. Clay minerals have been shown to have a strong binding capacity for large amounts of Cry3Bb1 proteins up to a range of milligram per gram [17]. As already reported for the Cry1Ab proteins from other *Bt* maize cultivars [46], the non-water extractable fraction of the soil adsorbed Cry3Bb1 can still maintain its full biological activity, as indicated by bioassays using a potato beetle [31]. In this study, extraction was done with PBST buffer, which is more efficient than water (data not shown). However, the low concentrations of Cry3Bb1 detected in rhizosphere soil in this study and the fact that there was no increase in detectable Cry3Bb1 during three years of subsequent MON88017 cultivation supports the conclusion that there is no accumulation of this protein in soil on agricultural fields, irrespective of its capacity to adsorb.

It is still debatable whether nanogram amounts of Cry-proteins detected in the rhizosphere of *Bt* maize are caused by root exudation [39,40], or whether they can be explained by sloughed root cells and small root hairs as contaminants of soil samples [9]. Considering the steep gradient between the concentrations of Cry3Bb1 proteins inside the roots with approx. 100 μg g^{-1} dw [31,34], and the lower than 1 ng g^{-1} soil amount detected in rhizosphere soil in this study, a contamination of 1 g of soil by only 10 μg of roots, which corresponds to a dilution of 1:100.000, would explain the amounts detected. Such low contamination by sloughed cells is probably unavoidable when separating the soil fraction from roots in the laboratory. Steep gradients in the amounts of Cry-proteins found in roots and rhizospheres of field grown plants have also been reported for MON810 with Cry1Ab in previous studies [4].

While the barrier between actively growing roots and surrounding soil seems extremely efficient, as demonstrated by the steep gradient of Cry3Bb1 between the inside and outside of the roots, the relatively large amounts of Cry3Bb1 inside the roots are potentially released into soil after harvest, when the maize stubbles remain on the field and the plant material begins to degrade. Initial concentrations could theoretically amount up to 2400 μg per kg of soil [9]. This study, however, demonstrates that degradation of Cry3Bb1 in decaying root residues on the agricultural fields are very high, starting from a maximum of 1684 ng Cry3Bb1 g^{-1} root residue, four weeks after harvest, down to 10 ng or less, 6 months later, and a further decline during the following growing season. Considering the initial concentrations of 100 μg Cry3Bb1 per g dw inside the roots, 10 ng Cry3Bb1 g^{-1} fw of degraded root material represent only 0.06%. In other studies it was demonstrated that Cry3Bb1 from decaying plant material did not leach into the mineral soil [36]. Furthermore, the overall decomposition of *Bt* maize (Cry3Bb1) under field conditions was shown to occur at the same rates as degradation of non-engineered varieties [29] and without any significant effects on the micro-, meso- and macrofauna involved [5,22,28].

The response of soil bacteria to MON88017 and Cry3Bb1 in this study was analyzed by cultivation independent analyses of their diversity. In the context of previous field studies with Cry3Bb1-expression *Bt* maize, microbiological analyses involving the quantification of soil microbial biomass and population sizes of culturable soil bacteria with different physiological potentials did not reveal any effect on the soil microbial community [23]. Here, the rhizosphere inhabiting bacterial community was analyzed, as it is known that plants have the capacity to select for specific soil bacteria by releasing nutrient sources and signal molecules [25]. Thus, it was expected that major modifications in the composition of the root exudates, such as a strong exudation of Cry3Bb1 proteins, would result in the selection of a differently structured community. Genetic profiling techniques based on PCR-amplified 16S rRNA genes from directly extracted soil DNA, i.e., TRFLP (terminal restriction fragment length polymorphism), DGGE (denaturing gradient gel electrophoresis), or SSCP (single strand conformation polymorphism) have previously been shown to be sensitive enough to detect effects of cultivars, plant age, site specific properties or other factors [11,30,32]. In contrast, there were no differences caused by the expression of Cry1Ab by the *Bt* maize MON810 [4]. This study also could not reveal any *Bt*-specific effect, even though effects of the recombinant Cry-protein were studied more thoroughly than previously by including group-specific PCR-primers. Thus, not only the diversity of the most dominant bacteria, but also of *Alphaproteobacteria* and *Pseudomonas* members was analyzed, the latter representing phylogenetic groups typically found in the rhizosphere of maize [8,37,42]. The lack of effect, which was also observed with MON863 with TRFLP in other studies [15] can be explained by the extremely low Cry3Bb1 concentrations found in the rhizosphere and otherwise obviously highly similar root properties of the maize varieties analyzed here.

In conclusion, the data of this study demonstrate that Cry3Bb1 proteins are unstable in plant residues and extractable Cry3Bb1 concentrations in soil from agricultural fields are extremely low with no accumulation during three subsequent years of *Bt* maize MON88017 cultivation. The fact that the diversity of soil bacteria in the immediate vicinity of *Bt* maize roots did not differ from that of other cultivars at the same growth stage and the strong degradation of Cry3Bb1 in root residues thus gives no indication of adverse effects caused by the genetically engineered properties on the soil ecology.

Acknowledgements

We thank Kathrin Ahrends, Britta Müller and Carsten Bitzer for their excellent technical assistance, Anja B. Dohrmann for advice and helpful comments on the manuscript, and members of the joint research project "Biosafety research on transgenic Diabrotica-resistant *Bt* maize", especially Sabine Eber, Sibylle Pagel-Wieder, Hang Thu Nguyen, Johannes Jehle, Jürgen Niemeyer, Ingolf Schuphan and Josef Wächter, for their support and discussion. The study was supported by grant 0313279F provided by the German Federal Ministry of Education and Research.

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Please cite this article in press as: R. Miethling-Graff et al., Release of the recombinant Cry3Bb1 protein of *Bt* maize MON88017..., Eur. J. Soil Biol. (2009), doi:10.1016/j.ejsobi.2009.10.003