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Assessing the risk posed to free-living soil nematodes by a genetically modified maize expressing the insecticidal Cry3Bb1 protein

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ABSTRACT

Before pest-resistant genetically modified maize can be grown commercially, the risks for soil-beneficial, non-target organisms must be determined. Here, a tiered approach was used to assess the risk to free-living soil nematodes posed by maize genetically modified to express the insecticidal Cry3Bb1 protein (event Mon88017), which confers resistance towards western corn rootworm (*Diabrotica virgifera*; Coleoptera). The toxicity of purified Cry3Bb1 for the nematode *Caenorhabditis elegans* was determined using a bioassay and gene expression analysis. In addition, a soil toxicity test was used to assess the effects on *C. elegans* of rhizosphere soil obtained from plots of an experimental field grown with Mon88017, the near-isogenic cultivar, or either of two conventional cultivars. Finally, the indigenous nematode communities from the experimental field site with Mon88017 and from the control cultivars were analyzed. The results showed a dose-dependent inhibitory effect of Cry3Bb1 on the growth and reproduction of *C. elegans*, with EC50 values of 22.3 mg l^{-1} and 7.9 mg l^{-1}, respectively. Moreover, Cry-protein-specific defense genes were found to be up-regulated in the presence of either Cry1Ab or Cry3Bb1. However, *C. elegans* was not affected by rhizosphere soils from Mon88017 compared to the control plots, due to the very low Cry3Bb1 concentrations, as indicated by quantitative analyses (<1 ng g^{-1} soil). Nematode abundance and diversity were essentially the same between the various maize cultivars. At the last sampling date, nematode genus composition in Bt-maize plots differed significantly from that in two of the three non-Bt cultivars, including the near-isogenic maize, but the shift in genus composition did not influence the composition of functional guilds within the nematode communities. In conclusion, the risk to free-living soil nematodes posed by Mon88017 cultivation can be regarded as low, as long as Cry3Bb1 concentrations in soil remain four orders of magnitude below the toxicity threshold.

1. Introduction

Bacillus thuringiensis (Bt)-crops provide a less harmful approach to pest control than conventional chemical strategies because of the pest-specific activity of their respective toxins (Cry proteins) and the inclusion of the insecticidal proteins within the crop during cultivation. Nonetheless, before pest-resistant genetically modified maize can be grown commercially, the risks for soil-beneficial, non-target organisms must be determined (Conner et al., 2003; Snow et al., 2005). Such studies have reported detrimental effects of Bt-maize expressing a truncated version of the insecticidal Cry1Ab protein, i.e., the variety Mon810, on non-target organisms (Hilbeck et al., 2002; Andow and Zwahlen, 2006), including nematodes (Manachini and Lozzia, 2002; Griffiths et al., 2005; Höss et al., 2008). In contrast to varieties of maize modified to express Cry1Ab, Cry3Bb1-expressing maize cultivars have been less intensively investigated under field conditions, especially in Europe, for their effects on non-target organisms (e.g., Al-Deeb et al., 2003; Li et al., 2008; Meissle and Romeis, 2009; Rauschen et al., 2009; Miethling-Graff et al., 2010). Cry3Bb1 is expressed in the transgenic maize event Mon88017 by a

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gene derived from *B. thuringiensis kumamotoensis* and it provides effective control of the corn pest *Diabrotica virgifera virgifera* (western corn rootworm) (Schnepf et al., 1998). This devastating pest has plagued maize cultivation throughout North America since 2003, leading to widespread commercial acceptance of *Diabrotica*-resistant Bt-maize varieties, i.e., those expressing Cry3Bb1, as an alternative to the use of insecticides (Hellmich et al., 2008). Moreover, in recent years, the western corn rootworm has also been introduced into Eastern and Central Europe and is now considered a serious threat to European corn yields (Miller et al., 2005).

Free-living, non-parasitic nematodes are the most abundant and species-rich metazoans in soils and are major contributors to their functioning (Yeates, 1981; Andrassy, 1992). With the evolution of several different feeding types, nematodes have successfully occupied key positions in terrestrial food webs (Yeates et al., 1993), thus influencing nutrient cycling in soils (Yeates and Coleman, 1982; Ingham et al., 1985; Beare, 1997). The presence of nematodes and the structure of nematode communities are therefore important to agricultural production and sustainability (Fiscus and Neher, 2002). Moreover, nematodes are suitable indicators for assessing soil pollution (Bongers, 1990; Nagy et al., 2004; Nagy, 2009).

For Cry1Ab-expressing maize, the protein was shown to enter the soil through root and plant residues (Zwahlen et al., 2003) or through feces deposited by animals that have fed on Bt-plant material (Weber and Nentwig, 2006; de Vaufleury et al., 2007). Consequently, free-living nematodes are exposed to Cry1Ab in soils where Bt-maize has been grown. However, direct effects can occur only if the protein concentrations in soil exceed a certain threshold, above which toxicity can be expected. For Cry3Bb1, there is as yet no indication that the protein persists or even accumulates in soil following its release by Bt-maize plants during cultivation or as a result of on-field decomposition (Ahmad et al., 2005; Icoz and Stotzky, 2008; Miethling-Graff et al., 2010).

Studies with single species of nematodes have demonstrated deleterious effects of *B. thuringiensis* isolates (Wharton and Bone, 1989; Meadows et al., 1990; Leyns et al., 1995; Borgonie et al., 1996; Belair and Cote, 2004), with specific classes of nematicidal Bt-toxins (Cry5, Cry6, Cry14, and Cry21) shown to have potent toxic effects on *Caenorhabditis elegans*, *Panagrellus redivivus*, and *Pristionchus pacificus* (Wei et al., 2003). These Cry-proteins affect nematodes by damaging the gut of the nematodes (Marroquin et al., 2000; Wei et al., 2003). Griffitts et al. (2003) showed that carbohydrate receptors, encoded by *bre* genes, on the intestinal cells of *C. elegans* interact with Cry proteins, allowing them to enter the cells and subsequently affect the nematode. The mode of action is, thus, analogous to that in insects (Schnepf et al., 1998). Although Cry1Ab concentrations above 0.7 µM (41 mg l^{-1}) were also shown to have toxic effects on nematodes, the protein's mode of action is not yet understood (Höss et al., 2008).

The aim of this study was to investigate the impact of *Diabrotica*-resistant maize (Mon88017) and, specifically, its insecticidal protein Cry3Bb1, on free-living nematodes in a tiered approach consisting of: (1) toxicity testing using purified Cry3Bb1 and the test organism *C. elegans*, (2) evaluation of the response of *C. elegans* to soil from field plots on which Mon88017 was cultivated, and (3) analysis of the effect of Mon88017 cultivation on the community structure of the indigenous, soil-inhabiting nematodes. In order to distinguish cultivar effects from those triggered by genetic modifications, comparisons were made with conventionally bred cultivars, among them the parental line of the Mon88017 event.

2. Materials and methods

2.1. Maize cultivars and field design

Bt-maize Mon88017 (Monsanto Company, St. Louis, MO, USA), the genetically modified maize variety used in the field experiment, expresses both the insecticidal Bt-toxin Cry3Bb1, due to genomic insertion of the *cry3Bb1* gene of *B. thuringiensis* ssp. *kumamotoensis*, and the glyphosate resistance protein CP4 EPSPS, encoded by the *cp4 epsps* gene of *Agrobacterium* sp. strain CP4. By expressing Cry3Bb1, the plant is protected against the western corn rootworm (*D. virgifera virgifera*, Coleoptera: Chrysomelidae).

Within an area of approximately 4 ha, four different maize lines were planted in a randomized complete block design (RCBD) with eight replicates each (Fig. 1). In order to distribute the various replicates of each maize line over the field, replicate plots were grouped in eight different blocks, each containing all four maize lines. Besides Bt-maize Mon88017 (Bt) and its near-isogenic line DKC5143 (Iso; both Monsanto Co.), two conventional maize lines, Benicia (Pioneer Hi-Bred, Johnston, IA, USA) and DK315 (Monsanto Co.), were used for the experiment. Individual plots measured 40.5 × 31.5 m (0.13 ha) and contained 42 rows of maize with 75 cm distance between them and 15 cm between individual plants. The plots were aligned in four parallel rows of eight plots each, with a 4.5-m-wide clearance between neighboring rows for easy access. The experimental field was surrounded by a 4.5-m clearance strip followed by a 10-m-wide perimeter of conventional maize (Gavott, KWS Saat, AG, Einbeck, Germany). The experiment was performed during three successive years, whereby the location of the plots with their respective maize lines was unchanged to ensure similar abiotic parameters. The field was planted on May 27 (calendar week (CW) 21) in 2005, on May 9 (CW 19) in 2006, and on May 21 (CW 21) in 2007. In 2005, a mixture of 0.8 l Motivell (BASF AG, active ingredient Nicosulfuron), 0.8 l Spectrum (BASF AG, active ingredient dimethenamid-p), and 2.0 l Artett (BASF AG, active ingredients terbuthylazine and bentazon) was used for weed control during the 2- to 8-leaf stadium. In 2006 and 2007, a mixture of 3.0 l Gardo Gold (Syngenta Co., active ingredients S-metolachlor and terbuthylazine) and 0.8 l Callisto (Syngenta Co., active ingredient mesotrione) was applied for this purpose.

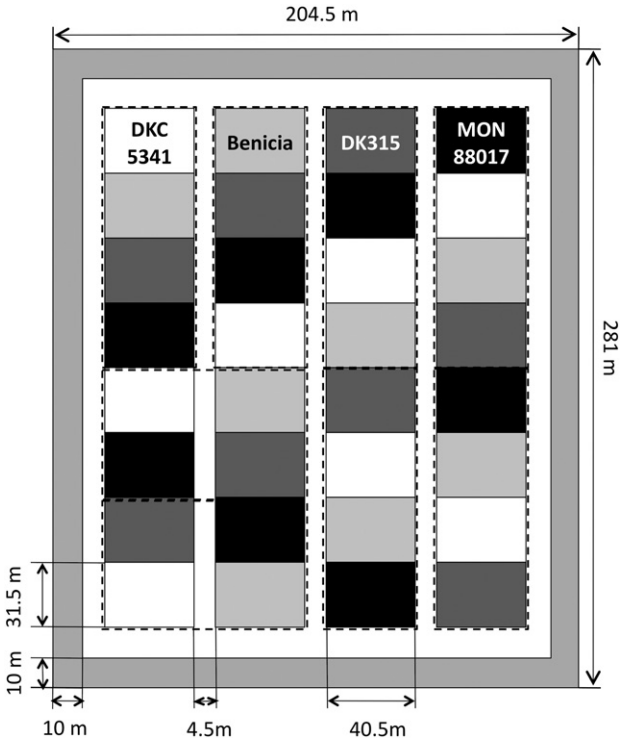

Fig. 1. Randomized complete block design (RCBD) with Bt-maize Mon88017, its near-isogenic line DKC5143, and the two conventional hybrids Benicia and DK315 planted in plots with eight replicates each; blocks ($n = 8$) are indicated by dashed lines; each maize cultivar is indicated by the cell shading; adapted from Rauschen et al., 2009.