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Earthworm populations in a northern U.S. Cornbelt soil are not affected by long-term cultivation of Bt maize expressing Cry1Ab and Cry3Bb1 proteins

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A B S T R A C T

Earthworms, which play a key role in biogeochemical processes in soil ecosystems, could be negatively affected by the cultivation of transgenic Bt crops. Studies to date have found few effects of Bt maize on earthworm species. If adverse effects occur, they are likely to be chronic or sub-lethal and expressed over large spatial and temporal scales. Our objective in the present study was to investigate potential effects on earthworm populations in soil cultivated with Bt maize in a large multiple-year field study. We surveyed the earthworm populations in 0.16-ha experimental field plots of two varieties of Cry1Ab Bt maize, one variety of Cry3Bb1 Bt maize, and three non-transgenic control varieties cultivated for four years. Four earthworm species were found in our sample: *Aporrectodea caliginosa*, *Aporrectodea trapezoides*, *Aporrectodea tuberculata* (collectively, the *A. caliginosa* species complex), and *Lumbricus terrestris*. We found no significant differences in the biomass of juveniles and adults for all four species between Bt and non-Bt maize varieties. From this and previous studies, we conclude that the effects of Cry1Ab and Cry3Bb1 Bt maize on the *A. caliginosa* species complex and *L. terrestris* are small. Nonetheless, general conclusions about the effects of Bt maize on earthworm populations are not warranted due to the small number of species tested. In future laboratory studies, earthworm species should be selected according to their association with a Bt crop and the impact of that species to valued soil ecosystem processes.

1. Introduction

Earthworms (Annelida: Oligochaeta) are a large and common component of soil biodiversity and, as “ecosystem engineers,” are one of the most important actors in the soil (Lavelle et al., 1997). In agricultural soils, earthworms are generally beneficial for plant growth and crop yield (Brown et al., 2004; Eriksen-Hamel and Whalen, 2007). The effects of different species of earthworms are related to their ecological functional guild, a classification scheme based on a species’ diet and its location in, and movement through, the soil layers (Bouché, 1977; Brown et al., 2004; Hale et al., 2005). Anecic species (e.g. *Lumbricus terrestris*) live in permanent vertical burrows that cross numerous soil layers and consume plant litter and partially decomposed organic matter, which they collect on the soil surface and carry down into their burrows. They facilitate water transport through soil layers (Shipitalo and Le Bayon, 2004) and the breakdown and incorporation of organic matter into soil (Satchell, 1983). Endogeic species (e.g. *Aporrectodea caliginosa*) live deep in soil, travel through temporary horizontal burrows, and consume organic matter that has already been incorporated into soil. Their actions aerate soil, facilitate horizontal water transport, and incorporate organic matter into the lower layers of soil (Fragoso et al., 1997). Epigeic species (e.g. *Eisenia fetida*) live within and consume leaf litter on the soil surface, facilitating initial breakdown of leaf litter (Fragoso et al., 1997; Hale et al., 2005).

Earthworm populations may be adversely affected by the cultivation of transgenic Bt crops, with negative consequences on soil quality through reduced earthworm activity (Mendonça-Hagler et al., 2006). Bt crops express one or several genes, originating from the bacterium *Bacillus thuringiensis*, for one or several proteins that are toxic to some important insect pests. These proteins are called Cry toxins and, of the several hundred different kinds produced by *B. thuringiensis*, Bt maize varieties may express Cry1Ab, Cry1A105, Cry2Ab2, Cry1F, mCry3A, Cry3Bb1, Cry34A/Cry35A, or some combination of these proteins. Bt maize and

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cotton are the only Bt crops commercially available in the USA currently; they were grown on about 57% of maize acreage and 63% of cotton acreage in the USA in 2008 (NASS, 2008).

Bt crops could adversely affect earthworm populations through direct toxicity of ingested Cry toxins, associated changes in the Bt crop, and/or associated changes in the soil flora and fauna (Birch et al., 2007). Earthworms may ingest Cry toxins from root exudates, clay particles in soil, and/or crop litter (Saxena and Stotzky, 2001b, Zwahlen et al., 2003b; Icoz and Stotzky, 2008; Pham et al., 2008).

Cry1Ab proteins from Bt maize (transformation events MON810 and Bt11) can persist in soil for at least 234 days when bound on clay particles (Tapp and Stotzky, 1998; Stotzky, 2004) and up to 2 years in Bt maize litter (Zwahlen et al., 2003a; Zwahlen and Andow, 2005). In contrast, Cry3Bb1 proteins from Bt maize (event MON863) degrade more rapidly: the proteins degraded in soil 50 days after being amended with Bt maize biomass in the laboratory (Icoz and Stotzky, 2007) and could not be detected in soils after three consecutive growing seasons in field trials (Ahmad et al., 2005; Icoz et al., 2008). Nonetheless, direct exposure of earthworms to Cry3Bb1 proteins may occur because of the high concentrations in Bt maize roots compared to roots of Cry1Ab Bt maize events (Clark et al., 2005), which may result in higher concentrations in root exudates and the rhizosphere.

Earthworms could also be affected by ecological changes associated with cultivation of a Bt crop (Latham et al., 2006; Hilbeck et al., 2008; Pham et al., 2008). For example, lignin levels in some but not all Cry1Ab Bt maize varieties are higher than in near-isogenic non-Bt varieties (Masoero et al., 1999; Escher et al., 2000; Saxena and Stotzky, 2001a; Jung and Sheaffer, 2004; Mungai et al., 2005; Poerschmann et al., 2005; Lang et al., 2006). Lignin is relatively indigestible, and the rate of decomposition is often inversely related to lignin:N ratios (Melillo et al., 1982; Taylor et al., 1989). Increased lignin content could contribute to observed reduced rates of Bt maize decomposition (Flores et al., 2005), although this was not confirmed in subsequent field studies (Cortet et al., 2006; Zwahlen et al., 2007; Tarkalson et al., 2008). Earthworms might also be affected by associated changes in microbial or faunal communities in soil (Hilbeck et al., 2008). On the other hand, the inconsistency of such changes and small effect sizes observed in empirical studies may indicate otherwise (Baumgarte and Tebbe, 2005; Griffiths et al., 2006, 2007a,b; Icoz and Stotzky, 2008; Icoz et al., 2008).

Previous studies on the effects of Bt crop varieties on earthworms suggest that no or low acute toxicity occurs to the earthworm species studied, but that sub-lethal adverse effects may occur in the field. The effects of Bt maize and Bt cotton cultivation on earthworms have, to date, been investigated in nine laboratory and four field studies (Ahl Goy et al., 1995; Saxena and Stotzky, 2001b; Zwahlen et al., 2003b; Clark and Coats, 2006; Ahmad et al., 2006; Vercesi et al., 2006; Lang et al., 2006; Krogh et al., 2007; Zwahlen et al., 2007; Hönemann et al., 2008; Schrader et al., 2008; Liu et al., 2009a,b). Of the laboratory studies, four tested the anecic species *L. terrestris* (Saxena and Stotzky, 2001b; Zwahlen et al., 2003b; Ahmad et al., 2006; Schrader et al., 2008), two tested the endogeic *A. caliginosa* species complex: *A. caliginosa*, *Aporrectodea nocturna*, *Aporrectodea trapezoides*, and *Aporrectodea tuberculata* (*sensu* Pérez-Losada et al., 2009 and references cited therein) (Vercesi et al., 2006; Schrader et al., 2008), and four tested the epigeic species *E. fetida* (Ahl Goy et al., 1995; Clark and Coats, 2006; Liu et al., 2009a,b). In their field studies, Lang et al. (2006) and Krogh et al. (2007) analyzed the abundances of one anecic species (*L. terrestris*), six endogeic species (including the *A. caliginosa* species complex), and one epigeic–anecic species (*Lumbricus rubellus*) in soil with Cry1Ab Bt maize. No studies have been published on the effects of Cry3Bb1 Bt maize on earthworm populations in the field. Zwahlen et al. (2007) and Hönemann et al. (2008) did not identify the earthworm species in their samples due to small sample sizes. All species in all studies were Lumbricid species native to Europe. Six laboratory and two field studies found no effect of Cry1Ab and Cry3Bb1 Bt maize on life-history parameters (survival, mortality, change in biomass, juvenile growth rate, and reproduction) or on abundance and biomass of field populations (Ahl Goy et al., 1995; Saxena and Stotzky, 2001b; Ahmad et al., 2006; Clark and Coats, 2006; Lang et al., 2006; Vercesi et al., 2006; Krogh et al., 2007; Schrader et al., 2008). Likewise, Liu et al. (2009a,b) found no observable acute effects from Cry1Ac Bt cotton on mortality and reproduction of *E. fetida*. In contrast, Clark and Coats (2006) found a slight positive effect on earthworm growth rate, and Zwahlen et al. (2003b) and Vercesi et al. (2006) found slight but statistically significant negative effects of Cry1Ab Bt maize. Zwahlen et al. (2003b) found that when adult *L. terrestris* were fed Bt maize in the laboratory, they grew slower than adults fed non-Bt maize, but this effect was observable only after 200 days. Vercesi et al. (2006) found a lower cocoon hatch rate for *A. caliginosa* var. *tuberculata* (Savigny) (=*A. tuberculata* Eisen).

Sub-lethal adverse effects on earthworms, such as those found by Zwahlen et al. (2003b) and Vercesi et al. (2006), may produce significant reductions in earthworm populations and, in turn, ecosystem services provided by them (Baveco and De Roos, 1996). Therefore, long-term studies are necessary to investigate if such effects may result in lower populations of earthworms in soils with Bt maize in the field (Zwahlen et al., 2003b). The objective of our study was to investigate the effects of one Cry3Bb1 and two Cry1Ab Bt maize varieties on the biomass of earthworm populations relative to near-isogenic non-transgenic maize varieties in a four-year, large-plot field study. We hypothesized that the cultivation of Bt maize reduces earthworm population biomass through one or more direct or indirect sub-lethal effect pathways. Any sub-lethal adverse effects on earthworm populations from Bt maize should be easier to detect in long-term field studies, with sufficient sample size, than in laboratory studies. The current literature on non-target effects from Bt crops has not provided clear and consistent indications of the mechanisms by which adverse effects may occur (Hilbeck and Schmidt, 2006), so testing a particular mechanistic hypothesis for our study is premature at this time. Any one of many direct or indirect ecological mechanisms (Andow et al., 2006) may result in reduced biomass of earthworm populations in soil cultivated with Bt maize, and any such reduction should be detectable in our study.

2. Materials and methods

2.1. Experimental design

We sampled earthworm populations in an experimental field at the UMore Park Research Station in Rosemount, Minnesota, USA (44°43′12″ N, 93°06′54″ W). Six maize varieties were planted in 40 m × 40 m plots in a complete randomized block design with four replicates, making a total of 24 plots. Plots were separated by a 20-m non-Bt maize buffer. Of the six maize varieties, three were Bt varieties: Cry1Ab Novartis N45-A6 (Bt11), Cry1Ab Pioneer 38A25 (MON810), and Cry3Bb1 DeKalb DKC46-24 (MON863). The Cry1Ab Bt varieties target the European corn borer, *Ostrinia nubilalis* Hübner [Lepidoptera: Crambidae], and the Cry3Bb1 Bt variety targets three species of corn rootworm, *Diabrotica* spp. [Coleoptera: Chrysomelidae]. The remaining three varieties were corresponding near-isogenic non-*Bt* varieties: Novartis N45-T6, Pioneer 38A24, and Dekalb DKC46-28. The same varieties were planted in the same plots for 4 growing seasons, from 2003 through 2006, except that