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Impact of coleopteran-active *Bt* corn on non-target nematode communities in soil and decomposing corn roots

Deborah A. Neher [a, *], Agnes W.N. Muthumbi [b], Galen P. Dively [c]

[a] Department of Plant and Soil Science, University of Vermont, 63 Carrigan Dr., Burlington, VT 05405, United States
[b] School of Biological Sciences, University of Nairobi, P.O. Box 30197-00100, Nairobi, Kenya
[c] Department of Entomology, University of Maryland, 4112 Plant Sciences Bldg., College Park, MD 20742, United States

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ABSTRACT

Genetically engineered corn expressing crystalline proteins for insect control and encoded by genes derived from soil bacterium *Bacillus thuringiensis* (*Bt*) are widely adopted in the United States. Among the seven different events of *Bt* corn available commercially, YieldGard® Rootworm (event MON863) expresses a variant of the *cry*3Bb1 protein in the root tissue to control corn rootworm larvae. Nematodes reside in the rhizosphere and are potentially exposed to Cry3Bb1 toxins exudated from roots of *Bt* corn. We test the hypothesis that coleopteran-active *Bt* corn does not affect non-target soil nematodes. Experimental treatments were: 1) a *Bt* hybrid, 2) a non-*Bt* isoline treated with a conventional soil insecticide, and 3) a non-*Bt* isoline without insecticide. Nematodes were extracted from soil samples collected prior to planting (May), at peak anthesis (August), and after harvest (October) in 2003 and 2004, enumerated and identified to genus. A total of 73 nematode genera were encountered in soil and litter combined. During the growing season, maturity index values and relative abundance of fungivorous nematodes were greater in the *Bt* hybrid than the non-*Bt* isoline with or without insecticide. Nematode trophic diversity values were greater in the *Bt* hybrid than non-*Bt* isoline with insecticide and this effect continued through the following spring. Abundance of nematode predators increased two weeks after insecticide was applied to non-*Bt* isoline, but decreased without insecticides on either *Bt* or the non-*Bt* isoline. In decaying roots of corn treatments, maturity index values and the relative abundance of nematode predators was greater in the *Bt* hybrid than non-*Bt* isoline with insecticide. Effects at the overall community structure and nematode genera varied more by seasonal phenology than corn treatment. The isoline with insecticide had more non-target effects on nematode communities than the *Bt* hybrid. This treatment increased the relative abundance of predaceous nematodes temporarily but eventually reduced successional maturity by harvest time, which continued to decline during the winter in both soil and decaying corn roots.

1. Introduction

Genetically engineered corn expressing proteins for insect control and encoded by genes derived from soil bacterium *Bacillus thuringiensis* (*Bt*) was grown on 88% of the national corn acreage in 2011 (USDA-Economic Research Service, 2012). Since the first introduction of *Bt* corn in 1996, there has been an increasing diversity of *Bt* transgenic events targeting broader pest populations and pyramided to express multiple insect-resistant traits. In 2003, Monsanto introduced the first coleopteran-active *Bt* corn (event MON863) expressing a variant of Cry3Bb1 protein in the root tissue to control corn rootworm larvae, *Diabrotica virgifera virgifera*. *Bt* corn is expected to pose little environmental impact due to the highly selective nature of the expressed proteins. Numerous laboratory tests have indicated no acute adverse effects on many non-target organisms and results of 47 field studies have shown no unexpected ecological risks to above-ground insect communities (in reviews by O'Callaghan et al., 2005; Romeis et al., 2006; Marvier et al., 2007; Wolfenbarger et al., 2008). However, fewer studies have addressed the possible impact of Cry proteins released from living or decaying roots of *Bt* corn on soil nematode communities, especially in field experiments. These semi-field or laboratory studies showed no or less impact of the lepidopteron-active Cry1Ab

* Corresponding author. Tel.: +1 802 656 0474.
E-mail addresses: deborah.neher@uvm.edu, dneher@uvm.edu (D.A. Neher), amuthumbi@uonbi.ac.ke (A.W. Muthumbi), galen@umd.edu (G.P. Dively).

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Table 1
Nematode genera present in at least 5% of the study samples of soil and litter for Bt corn (event MON 863 YieldGard® Rootworm) expressing the Cry3Bb1 protein (*Bt*), non-*Bt* isoline without insecticide (Iso − I) as a negative control, and non-*Bt* isoline with a soil insecticide tefluthrin (Iso + I) as a positive control.

Genus	CP[a]	Soil (no. per gram dry soil)			Corn roots (no. per 0.1 gram dry root)		
		Bt	Iso − I	Iso + I	*Bt*	Iso − I	Iso + I
1. Bacterivores		**2.80 ± 0.1**	**3.70 ± 0.2**	**3.31 ± 0.2**	**83.492 ± 10.94**	**1053.24 ± 202.7**	**980.73 ± 155.6**
Acrobeles	2	0.46 ± 0.04	0.53 ± 0.06	0.32 ± 0.04	12.74 ± 4.58	5.01 ± 1.38	4.23 ± 1.10
Acrobeloides	2	0.03 ± 0.01	0.06 ± 0.01	0.08 ± 0.03	11.13 ± 2.32	7.91 ± 3.06	6.33 ± 1.94
Alaimus	4	0.08 ± 0.01	0.09 ± 0.01	0.07 ± 0.01	1.53 ± 0.70	2.56 ± 1.08	0.76 ± 0.40
Anaplectus	2	0.18 ± 0.02	0.19 ± 0.03	0.11 ± 0.01	4.42 ± 1.21	4.95 ± 1.31	4.67 ± 2.35
Bastiania	3	0.05 ± 0.01	0.08 ± 0.01	0.04 ± 0.01	1.54 ± 0.68	1.06 ± 0.55	0.28 ± 0.19
Bunonema	1	0	0	0	1.38 ± 0.60	0.17 ± 0.10	0.22 ± 0.18
Cephalobus	2	0.44 ± 0.05	0.38 ± 0.04	0.50 ± 0.08	110.84 ± 20.58	112.42 ± 34.26	59.91 ± 18.43
Cervidellus	2	0.16 ± 0.03	0.09 ± 0.02	0.07 ± 0.01	0	0	0
Cruznema	1	0.06 ± 0.01	0.05 ± 0.02	0.06 ± 0.02	0	0	0
Cylindrolaimus	3	0.08 ± 0.02	0.10 ± 0.02	0.15 ± 0.04	2.69 ± 0.98	3.28 ± 1.40	0.79 ± 0.41
Diplogasteriana	1	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	1.89 ± 0.95	3.73 ± 1.52	1.13 ± 0.65
Diplolaimelloides	2	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.01	0	0	0
Diploscapter	1	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.01	353.64 ± 85.86	548.28 ± 131.13	551.48 ± 122.89
Eucephalobus	2	0.12 ± 0.02	0.21 ± 0.02	0.25 ± 0.04	95.68 ± 15.19	62.60 ± 10.91	60.47 ± 11.20
Eumonhystera	2	0.08 ± 0.02	0.07 ± 0.02	0.09 ± 0.03	28.44 ± 7.28	35.42 ± 10.31	30.84 ± 12.14
Macrolaimellus	2	0.02 ± 0.01	0.04 ± 0.01	0.02 ± 0.01	0	0	0
Mesorhabditis	1	0.06 ± 0.01	0.11 ± 0.02	0.05 ± 0.02	3.02 ± 1.73	5.48 ± 4.16	1.27 ± 0.54
Metateratocephalus	3	0.02 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.38 ± 0.24	1.66 ± 0.78	1.29 ± 0.67
Monhystera	2	0.12 ± 0.03	0.19 ± 0.03	0.19 ± 0.03	4.04 ± 1.80	10.84 ± 4.07	10.33 ± 5.19
Odontolaimus	3	0.01 ± 0.00	0.01 ± 0.00	0	0	0	0
Panagrolaimus	1	0.03 ± 0.01	0.02 ± 0.01	0.08 ± 0.03	12.51 ± 4.63	21.69 ± 6.68	17.88 ± 6.24
Paramphidelus	4	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0	0	0
Plectus	2	0.12 ± 0.02	0.31 ± 0.04	0.23 ± 0.03	83.76 ± 20.79	64.34 ± 15.24	54.90 ± 20.72
Prismatolaimus	2	0.22 ± 0.03	0.41 ± 0.06	0.36 ± 0.04	3.19 ± 1.43	4.00 ± 1.42	10.61 ± 4.03
Protorhabditis	1	0.01 ± 0.00	0.01 ± 0.01	0.02 ± 0.01	33.85 ± 11.52	51.44 ± 21.86	68.21 ± 26.13
Pseudacrobeles	2	0.05 ± 0.03	0.06 ± 0.01	0.04 ± 0.01	32.53 ± 6.73	25.25 ± 5.41	29.42 ± 11.27
Rhabditis	1	0.24 ± 0.04	0.31 ± 0.13	0.31 ± 0.03	34.85 ± 10.61	82.13 ± 42.11	63.12 ± 17.90
Rhabdolaimus	2	0.01 ± 0.00	0.06 ± 0.01	0.05 ± 0.01	0	0	0
Teratolobus	2	0.01 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	1.47 ± 0.78	0.84 ± 0.50	0.56 ± 0.30
Theristus	2	0.01 ± 0.00	0.01 ± 0.00	0	0	0	0
Tylocephalus	2	0.03 ± 0.01	0.08 ± 0.02	0.05 ± 0.01	0.56 ± 0.32	1.41 ± 0.54	0.52 ± 0.22
Wilsonema	2	0.02 ± 0.01	0.08 ± 0.02	0.05 ± 0.01	0.73 ± 0.31	0.49 ± 0.19	2.65 ± 1.06
Zeldia	2	0.01 ± 0.00	0.01 ± 0.00	0	0	0	0
2. Fungivores		**1.26 ± 0.1**	**0.99 ± 0.1**	**1.01 ± 0.10**	**183.16 ± 23.9**	**262.40 ± 48.1**	**238.48 ± 44.5**
Aphelenchoides	2	0.50 ± 0.07	0.35 ± 0.05	0.41 ± 0.07	138.62 ± 20.00	205.84 ± 40.83	198.18 ± 41.56
Aphelenchus	2	0.12 ± 0.02	0.10 ± 0.02	0.09 ± 0.02	2.19 ± 0.63	2.34 ± 1.00	1.61 ± 0.71
Diphtherophora	3	0.16 ± 0.03	0.14 ± 0.02	0.11 ± 0.02	0	0	0
Ditylenchus	2	0.16 ± 0.03	0.08 ± 0.01	0.10 ± 0.02	12.67 ± 4.03	11.18 ± 2.31	11.54 ± 2.68
Filenchus	2	0.22 ± 0.02	0.21 ± 0.02	0.24 ± 0.03	9.53 ± 3.60	21.25 ± 8.25	15.01 ± 3.42
Leptonchus	4	0.03 ± 0.01	0.04 ± 0.01	0.02 ± 0.01	0	0	0
Longidorella	4	0	0.02 ± 0.01	0.01 ± 0.00	2.43 ± 1.04	3.41 ± 1.47	1.32 ± 0.58
Paraphelenchus	2	0.01 ± 0.00	0	0	0	0	0
Tylencholaimus	4	0.05 ± 0.01	0.05 ± 0.01	0.02 ± 0.01	17.72 ± 3.95	18.37 ± 6.15	10.82 ± 2.87
3. Plant-parasites		**2.58 ± 0.2**	**3.05 ± 0.3**	**2.75 ± 0.2**	**18.23 ± 4.0**	**19.13 ± 6.2**	**11.82 ± 3.3**
Basiria	2	0.01 ± 0.00	0.01 ± 0.00	0.04 ± 0.01	0	0	0
Boleodorus	2	0	0	0.02 ± 0.01	0	0	0
Coslenchus	2	0.07 ± 0.03	0.10 ± 0.02	0.05 ± 0.02	0	0	0
Ecphyadophora	2	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0	0	0
Helicotylenchus	3	0.22 ± 0.07	0.48 ± 0.15	0.25 ± 0.04	0	0	0
Hoplolaimus	3	0.25 ± 0.03	0.19 ± 0.03	0.24 ± 0.03	0	0	0
Meloidogyne	3	0.01 ± 0.01	0.01 ± 0.00	0	0	0	0
Paratrichodorus	4	0.11 ± 0.02	0.07 ± 0.01	0.11 ± 0.02	0	0	0
Pratylenchus	3	0.63 ± 0.10	0.45 ± 0.06	0.36 ± 0.05	0	0	0
Pungentus	4	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0	0	0
Trichodorus	4	0.01 ± 0.00	0	0	0	0	0
Tylenchorhynchus	2	1.23 ± 0.16	1.70 ± 0.18	1.64 ± 0.20	18.23 ± 3.95	19.13 ± 6.22	11.82 ± 3.26
4. Omnivores		**0.98 ± 0.1**	**1.07 ± 0.1**	**0.67 ± 0.1**	**33.10 ± 5.6**	**37.66 ± 7.3**	**25.63 ± 4.3**
Achromadora	3	0.21 ± 0.03	0.30 ± 0.05	0.18 ± 0.02	4.30 ± 1.67	3.94 ± 0.97	5.34 ± 2.09
Aporcelaimellus	5	0.12 ± 0.02	0.10 ± 0.02	0.03 ± 0.01	3.87 ± 1.54	1.18 ± 0.57	1.03 ± 0.44
Dorylaimellus	5	0	0.01 ± 0.00	0	0	0	0
Eudorylaimus	4	0.16 ± 0.03	0.18 ± 0.03	0.09 ± 0.02	9.48 ± 2.27	11.46 ± 3.84	5.16 ± 2.33
Glauxinemella	1	0.08 ± 0.03	0.02 ± 0.01	0.05 ± 0.02	0	0	0
Laimydorus	4	0.14 ± 0.01	0.19 ± 0.02	0.12 ± 0.02	0	0	0
Lordellonema	4	0	0.03 ± 0.01	0	0	0	0
Mesodorylaimus	4	0.10 ± 0.01	0.08 ± 0.02	0.11 ± 0.02	10.94 ± 3.70	16.06 ± 4.65	10.92 ± 3.03
Pristionchus	1	0.07 ± 0.02	0.04 ± 0.01	0.03 ± 0.01	0.40 ± 0.20	0	1.12 ± 0.54
Thornenema	4	0.10 ± 0.02	0.14 ± 0.02	0.06 ± 0.02	2.22 ± 0.66	1.29 ± 0.46	0.95 ± 0.41

Table 1 (*continued*)

Genus	CP[a]	Soil (no. per gram dry soil)			Corn roots (no. per 0.1 gram dry root)		
		Bt	Iso − I	Iso + I	*Bt*	Iso − I	Iso + I
5. Predators		**0.25 ± 0.0**	**0.28 ± 0.0**	**0.15 ± 0.0**	**7.61 ± 1.4**	**9.84 ± 2.7**	**3.93 ± 1.1**
Clarkus	4	0.08 ± 0.02	0.07 ± 0.01	0.06 ± 0.01	6.02 ± 1.16	8.73 ± 2.72	3.44 ± 1.05
Discolaimus	4	0.06 ± 0.01	0.04 ± 0.01	0.04 ± 0.00	0	0	0
Ironus	4	0.01 ± 0.00	0.01 ± 0.00	0	0	0	0
Mylonchulus	4	0.03 ± 0.01	0.04 ± 0.01	0.02 ± 0.01	0	0	0
Nygolaimus	5	0	0.02 ± 0.01	0	0	0	0
Paraxonchium	5	0	0.01 ± 0.00	0.01 ± 0.00	0	0	0
Seinura	2	0	0	0	1.59 ± 0.81	1.11 ± 0.43	0.49 ± 0.29
Solididens	5	0	0	0	0	0	0
Tripyla	3	0.06 ± 0.01	0.09 ± 0.02	0.01 ± 0.00	0	0	0
Total nematodes		**6.13 ± 0.4**	**9.85 ± 0.5**	**9.41 ± 0.6**	**1096.1 ± 128.8**	**1415.1 ± 252.0**	**1295.30 ± 195.6**

[a] Colonizer-persister value (Bongers, 1990; Bongers et al., 1991, 1995), based on life history characteristics on a scale ranging from 1 to 5, with 1 representing *r*-strategists and 5 representing *K*-strategists.

protein on microbial community structure compared to disturbances from other environmental factors, such as plant age or heterogeneity of field properties (Blackwood and Buyer, 2004; Devare et al., 2004; Baumgarte and Tebbe, 2005; Fang et al., 2005; Höss et al., 2011). Furthermore, the addition of soil insecticide had greater effects on microbial function in soil and decaying roots than Cry3Bb1 *Bt* corn (Lawhorn et al., 2009).

Laboratory studies have demonstrated that different *Bt* toxins (Cry5B, Cry6A, Cry14A, and Cry21A) have deleterious effects on four bacterivorous species of nematodes (Wei et al., 2003). There is also soil bioassay evidence that Cry1Ab and Cry3Bb1 toxins, at higher than field relevant doses, have an inhibitory effect on the growth and reproduction in *Caenorhabditis elegans* (Höss et al., 2008, 2011). These Cry proteins apparently affect nematodes by binding to specific receptors on the epidermal wall of the gut, similar to that in insects, although the mode of action is not fully understood (Wei et al., 2003). However, field studies have reported differing effects of *Bt* transgenic crops on nematodes. Al-Deeb et al. (2003) and Höss et al. (2011) concluded that nematode abundance and functional diversity were not significantly affected in rhizosphere soil of MON88017 or MON863 Bt corn. A more recent study (Karuri et al., 2013) also reported that *Bt* cotton containing Cry1Ac and Cry2Ab2 protein had no significant effect on nematode diversity.

A remaining question is whether nematicidal effects are also observed in the field with soils naturally containing mixtures of nematode taxa. One report suggests total abundance of soil nematodes was similar in coleopteran-active *Bt* corn and non-*Bt* corn fields in a 2-year study (Al-Deeb et al., 2003). However, the non-target effects of *Bt* corn hybrids expressing the coleopteran-active proteins on non-target soil nematodes have not been evaluated at the genus level in a field study. In this study, we focused on the non-target effects of MON863 (YieldGard® Rootworm) *Bt* corn on the soil nematode community, therefore exploring the potential ecotoxicological risk of *Bt* proteins to agricultural ecosystems. We tested the hypothesis that coleopteran-active *Bt* corn does not affect non-target nematodes in the rhizosphere or decaying roots. We also predicted that any non-target effect on nematodes would be short-lived, lasting less than one growing season.

2. Methods

2.1. Field site

The experimental site was a 5.4-ha section of a field consisting of six contour strips, each 30 m wide by 300 m long, located at the University of Maryland Research and Education Center, Beltsville, MD (39.034°N, 76.907°W). The site was slightly sloped (mean 5 degree grade running perpendicular to the contour strips) and surrounded by woodlots on all four sides. Soil types consisted of Sunnyside fine sandy loam and Galestown–Evesvoro loamy sand, with mean (±1 SD) pH and organic matter of 6.0 (±0.2) and 1.2% (±0.2), respectively. Previous cropping practices consisted of alternating strips of non-transgenic corn and soybean under no-tillage cultivation.

2.2. Treatments

In 2003, three corn treatments arranged in a Latin square design were established in plots measuring 30 by 90 m within the first, third, and fifth contour strips. The treatments were: 1) *Bt* corn (hybrid DKC 61-44RR; event MON 863) expressing the Cry3Bb1 protein; 2) non-*Bt* near-isoline treated with a soil insecticide teflu-thrin (Force®, Syngenta Crop Protection, Raleigh, NC), as a positive control; and 3) the untreated, non-*Bt* near-isoline as a negative control. The remaining adjacent strips (second, fourth, and sixth) were planted with soybean. Treatment plots within each strip were planted side-by-side without buffers. In 2004, treatment plots were arranged in the same design but planted in the second, fourth and sixth strips of surface residue remaining from the previous year's soybean crop. In 2005, plot layout and contour strips used in 2003 was repeated. During each year, plots were planted no-till in early May and managed according to recommended fertility and herbicide regimes. Force was applied as a granule insecticide in the seed furrow at planting time at the rate of 5 kg per hectare. Each year represented a replicated block of the entire experiment.

2.3. Data collection

2.3.1. Corn roots

Saran mesh bags filled with root tissue were used as an in-field assay to assess if the treatments affected nematode colonization of decaying roots in soil. Roots were collected at anthesis in each treatment plot, washed free of soil, and coarsely mulched into smaller pieces during August 2003 and July 2004. During each year, 22 litter bags (26 cm 14 cm; 1 mm by 1.5 mm mesh size) containing 100 g of root tissue were buried in the soil (10-cm depth) within the central area of each plot. Four bags were removed at 1, 2, 3, 8, and 9 months later from each plot and root tissue from pairs of bags were combined into two composite subsamples. Ten grams of tissue was randomly collected from each subsample and placed in an intermittent misting chamber for 3 days to extract nematodes (Seinhorst, 1950). An additional 20 samples of *Bt* and non-*Bt* root material were weighed and then dried to estimate a wet to dry weight conversion factor. Abundance of nematodes was expressed as number per gram of dry corn root tissue.

Soil samples were collected at three crop phenology times (planting, anthesis, harvest) during 2003 in 2004 by following a x-pattern through the central area of each plot and taking 20 cores with a core tube (2-cm in diameter and 10-cm deep) (Oakfield Apparatus Co., Oakfield, Wisconsin, USA). All core samples were mixed and homogenized by hand to form a composite sample, and then further sub-divided into two subsamples. Similar subsamples were collected during the spring of 2004 and 2005 in each plot prior to soybean planting to assess carryover treatment effects. Nematodes were extracted from each subsample using Cobb's decanting and sieving with cotton milk filter trays (Whitehead and Hemming, 1965) immediately upon arrival to the laboratory. Additional samples taken from the same plot were dried at 55 °C to provide the dry weight to determine gravimetric moisture. Abundance of nematodes was expressed as number per gram of dry soil.

An inverted microscope was used for enumerating nematodes from a 10% sub-sample of each extract, and the nematodes were heat-fixed in formalin and stored in vials for later identification. A compound-light microscope was used for identifying and enumerating nematodes by taxonomic genus according to Andrássy (1983), Bongers (1987), Maggenti et al. (1987), Jairajpuri and Ahmad (1992), Hunt (1993), Siddiqi (2000), and DeLey et al. (2001). Taxonomic families were assigned to trophic groups according to Yeates et al. (1993). Families of nematodes were assigned CP values (Bongers, 1990; Bongers et al., 1991, 1995), based on life history characteristics on a scale ranging from 1 to 5, with 1 representing *r*-strategists and 5 representing *K*-strategists (Table 1). Permanent mounts were made in anhydrous glycerol (S'Jacob and van Bezooigen, 1984) and voucher specimens preserved in 10% formalin and 1.0% glycerin, sealed with parafilm (Neher and Campbell, 1994; Neher et al., 1998).

2.3.2. Community structure

Indices were estimated of tropic diversity, generic diversity, and successional maturity indices of nematode communities (plant-parasitic and free-living). As a measure of food web complexity, trophic diversity Hills N1 index was computed as $\exp - \sum [P_i (\ln P_i)]$ where P_i is the proportion of trophic group i in the total nematode community and reflects the number of abundant trophic groups (N1 is eH′ where e is the natural log and H′ is Shannon index (Neher and Darby, 2006)). Hills indices are simpler to interpret ecologically than commonly used Shannon forms. Successional maturity indices were computed two ways, i.e., free-living nematodes with CP1 through CP5 (MI), plant-parasitic nematodes (PPI). Maturity indices are weighted means computed as Σ [CP-value (i) *f(i)]/[total numbers of nematodes] where (i) is the individual taxon and f(i) is the frequency of the taxa in a sample (Bongers, 1990). Three extensions of the maturity index were also computed, i.e., channel index (CI), enrichment index (EI), and structural index (SI) (Ferris et al., 2001).

2.4. Statistical analysis

Means and standard errors were computed for each nematode genus present in at least 5% of the soil and litter samples. A mixed model ANOVA (SAS Release 9.3, SAS Institute Inc., Cary, North Carolina, USA) was performed to test for significant treatment effects on each index of the abundance and community composition of nematodes. Averages of two subsamples were analyzed. The repeated measures option was used to adjust for autocorrelation effects among sampling dates. LSMEANS with Tukey's adjustment was used to test for differences among combinations of the treatments. Random effects were column and row (Latin Square) and year. All variables were tested for normality prior to analysis using the Univariate procedure. Abundance per gram of dry soil were transformed as ln ($x + 0.1$) and abundance per gram of dry root were transformed as ln ($x + 1$). The difference in constants reflects the order of magnitude difference in abundance for the two substrates. Relative abundance in each trophic group was transformed as the arcsine of the square root of the proportion of total abundance residing in a trophic group.

Specific ANOVA models were tested to address each of following four questions: 1) Are there differences in nematode community composition in response to the corn treatments and the cropping year with crop phenology (planting, anthesis, harvest) as a repeated measure? This analysis included 54 samples (3 treatments × 3 crop phenology points × 2 years × 3 replicates); 2) Do effects of the treatments carryover to the following spring? In this analysis, a total of 36 samples consisted of 3 treatments × 2 times (corn harvest, soybean planting the following spring) × 2 years × 3 replicates; 3) Are effects apparent two weeks after planting that disappear by anthesis? This involved 3 treatments × 2 times × 3 replicates (0 and 2 weeks after planting) for a total of 18 samples; and 4) Does corn treatment affect colonization of decaying roots in soil? Corn roots were buried at anthesis and sampled at 1, 2, 3, 8, and 9 months later, for a total of 90 samples (3 treatments × 5 months × 2 years × 3 replicates).

Partial Redundancy Analysis (RDA) was performed as a multivariate approach to test for treatment effects on all nematode genera as a community using Canoco software, version 5 (Microcomputer Power, Ithaca, New York, United States). RDA was chosen because response data have a gradient 1.6 SD units long suggesting a linear method. Response data were log-transformed and genera center and standardized. Year was included as a co-variable and treated as a block. Significance was determined after permutations as a split design with treatments (whole plots) freely exchangeable and phenology (subplots) not permutated. This allows the three treatments and three replicates to be shuffled at random within year. *P*-values are adjusted for false discovery rate and, thus, conservative.

3. Results

A total of 73 nematode genera were encountered in soil and litter combined (Table 1), and numbers of nematodes were greater in May and October than August. Of these 33, 9, 12, 10, and 9 genera of bacterivores, fungivores, plant-parasites, omnivores, and predators were enumerated, respectively. Of the trophic groups, bacterivores were most abundant, followed progressively by fungivores, omnivores, plant-parasites, and predators. The most abundant genus was *Tylenchorynchus*, which accounted for 25% of the total abundance.

3.1. Soil community

The two-way interaction effect of treatment and crop phenology was not significant for all indices of the abundance and community composition of soil nematodes. However, corn treatment significantly affected the free-living maturity index (MI) and the proportion of fungivores but not total abundance, bacterivores, predators, omnivores, N1, EI, CI, or SI (Table 2). There also were crop phenology effects on MI, plant-parasites, fungivores, omnivores, and N1 but not total abundance, bacterivores, predators, PPI, CI, EI or SI (Table 2). MI values were smaller in the isoline with insecticide compared to *Bt* (Fig. 1a), although this main affect was primarily due to the difference at planting. Proportion of fungivores was significantly greater in *Bt* plots at all phenology stages than the proportion present in isoline plots without insecticide (Fig. 1b). The RDA analysis showed that the abundances of nematode genera as a whole community were influenced significantly by the interaction

Table 2
Two-way repeated measures ANOVA on nematode communities in soils with corn treatments (Bt, Isoline − insecticide, Isoline + insecticide). Phenology (planting, anthesis, harvest) was treated as a repeated measure, adjusted for within-subject correlation using an autoregressive structure. Sample size was 54 per treatment after averaging subsamples (3 treatments × 3 phenology times × 2 years × 3 replicates). Year, row and column of the Latin square design were treated as random variables.

Index	Treatment (df = 2)		Phenology (df = 2)		Treatment × Phenology (df = 4)	
	F-values	*P*-values	*F*-values	*P*-values	*F*-values	*P*-values
ln (density)[a]	0.36	0.6991	0.34	0.7115	0.33	0.8592
PPI[b]	2.40	0.1034	2.41	0.1024	1.71	0.1669
MI[b]	4.36	0.0191	6.80	0.0028	1.65	0.1803
Plant-Parasites[c]	0.72	0.4948	3.82	0.0300	1.30	0.2861
Fungivores[c]	4.44	0.0179	10.56	0.0002	0.21	0.9290
Omnivores[c]	2.33	0.1094	5.67	0.0066	1.66	0.1774
Bacterivores[c]	0.95	0.3966	2.40	0.1032	1.64	0.1827
Predators[c]	0.12	0.8848	1.87	0.1665	0.38	0.8247
N1trophic[d]	1.94	0.1562	3.28	0.0473	1.20	0.3244
CI[e]	1.86	0.1681	1.67	0.1997	0.37	0.8270
EI[e]	1.64	0.2055	1.66	0.2026	0.27	0.8946
SI[e]	0.98	0.3848	2.68	0.0802	0.41	0.8010

[a] Transformed as ln (x + 0.1).
[b] Successional maturity indices of nematode communities: PPI (plant-parasitic nematodes cp 2 to cp5), MI (free-living nematodes cp1–cp5), Maturity indices are weighted means computed as Σ [CP-value (i)*f(i)]/[total numbers of nematodes] where (i) is the individual taxon and f(i) is the frequency of the taxon in a sample (Bongers, 1990).
[c] Trophic groups of nematodes, transformed as arcsine of the square root.
[d] Trophic diversity: exp − ∑ [P_i (ln P_i)] where P_i is the proportion of group (trophic level) i in the total nematode community and reflects food web complexity.
[e] Channel index (CI), enrichment index (EI), and structural index (SI) (Ferris et al., 2001).

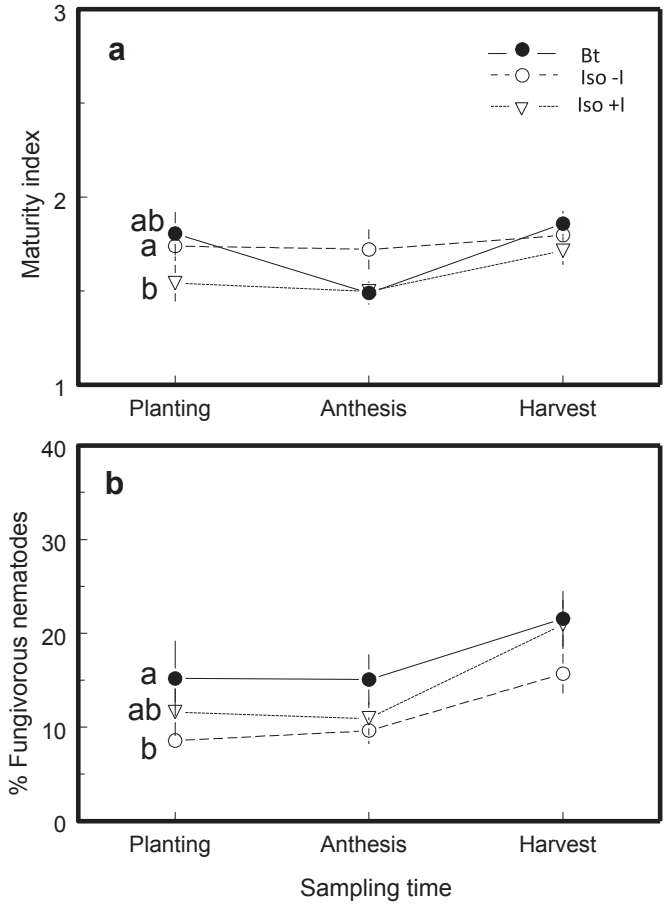

Fig. 1. Corn treatment effects on a) maturity index of free-living nematodes and b) percentage of fungivores across crop phenology (planting, anthesis, harvest). Arithmetic means ± 1 SE are illustrated for *Bt* hybrid (Bt), non-*Bt* isoline without insecticide (Iso − I), and non-*Bt* isoline with insecticide (Iso + I). Sample size was 54 after averaging subsamples (3 treatments × 3 phenology times × 2 years × 3 replicates). Contrasting letters represent statistical differences ($p \leq 0.05$).

of hybrid treatment and crop phenology but more variability was explained by phenology than treatments (Fig. 2). This is indicated by the treatment vectors grouped by phenology and pointed in the direction of the same nematode genera. Before corn treatments were planted, there was some variation among the soil nematode community characterized mostly by omnivores *Glauxinemella* and *Aporcelaimellus*, bacterivores *Rhabditis* and *Pseudacrobeles*, and plant-parasites *Pratylenchus*, *Paratrichodorus*, and *Hoplolaimus*. Plant-parasite *Basiria* was more abundant in isoline plots treated with insecticide and this accounted for most of the differences at anthesis. A contrasting combination of bacterivores (e.g., *Bastiania*, *Macrolaimellus*, *Diplolaimelloides*, *Acrobeles*, *Rhabdolaimus*,

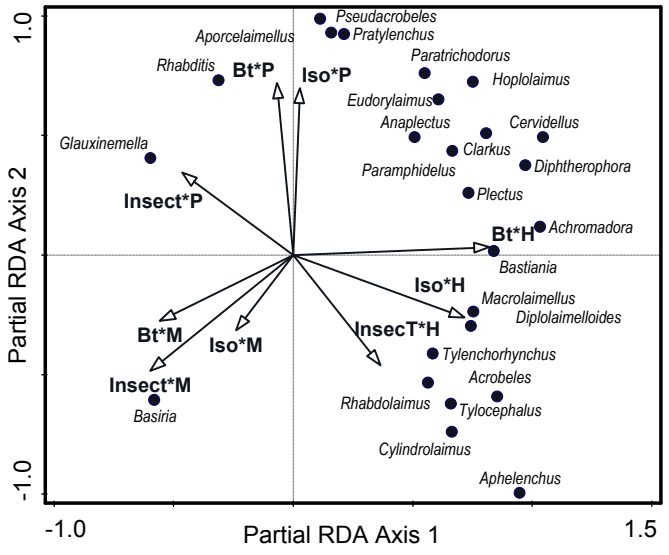

Fig. 2. Constrained-Partial redundancy analysis (RDA) biplot of nematode genera in soil with the two-way interaction of treatment and phenology as explanatory variables and year as a covariable treated as a block (Bt: *Bt* hybrid, Iso: non-*Bt* isoline without insecticide, Insect: non-*Bt* isoline with insecticide tefluthrin; P: pre-plant, M: mid-season at anthesis, and H: harvest phenology). Circles represent each of the 25 of 73 nematode genera that explained the most variation. Eigenvalues (lambda) are 0.0785 (pseudo-F = 4.7, P = 0.006), 0.0504, 0.0223, and 0.0142 for the first (horizontal), second (vertical), third and fourth axes respectively. The first two axes represent 64.48% of the fitted variation. Sample size was 18 per treatment after averaging subsamples (3 phenology times × 2 years × 3 replicates).

Table 3
Two-way ANOVA on the effect of overwintering (fall harvest, spring pre-plant) effect on nematode communities in soil with corn treatments (Bt, Isoline − insecticide, Isoline + insecticide). Sample size was 36 per treatment after averaging subsamples (3 treatments × 2 phenology times × 2 years × 3 replicates). Year, row and column of the Latin square design were treated as random variables.

Index	Treatment (df = 2)		Phenology (df = 1)		Treatment × Phenology (df = 2)	
	F-values	*P*-values	*F*-values	*P*-values	*F*-values	*P*-values
ln (density)[a]	0.10	0.9015	0.03	0.8657	0.01	0.9927
PPI[b]	1.46	0.2502	9.75	0.0042	0.12	0.8855
MI[b]	1.94	0.1633	7.83	0.0094	0.22	0.8020
Plant-Parasites[c]	0.53	0.5964	0.67	0.4189	0.87	0.4323
Fungivores[c]	2.93	0.0708	17.90	0.0002	0.14	0.8728
Omnivores[c]	0.96	0.3941	0.31	0.5836	0.30	0.7424
Bacterivores[c]	1.83	0.1805	0.13	0.7217	1.46	0.2505
Predators[c]	1.94	0.1635	2.57	0.1208	0.42	0.6593
N1trophic[d]	4.21	0.0256	5.89	0.0221	1.08	0.3527
CI[e]	0.99	0.3825	0.86	0.3616	1.27	0.2984
EI[e]	2.59	0.0933	8.72	0.0064	0.28	0.7564
SI[e]	0.70	0.5070	0.67	0.4192	0.18	0.8334

[a] Transformed as ln $(x + 0.1)$.
[b] Successional maturity indices of nematode communities: PPI (plant-parasitic nematodes cp 2 to cp5), MI (free-living nematodes cp1−cp5), Maturity indices are weighted means computed as Σ [CP-value (i)*f(i)]/[total numbers of nematodes] where (i) is the individual taxon and f(i) is the frequency of the taxon in a sample (Bongers, 1990).
[c] Trophic groups of nematodes, transformed as arcsine of the square root.
[d] Trophic diversity: exp − ∑ $[P_i (\ln P_i)]$ where P_i is the proportion of group (trophic level) i in the total nematode community and reflects food web complexity.
[e] Channel index (CI), enrichment index (EI), and structural index (SI) (Ferris et al., 2001).

Tylocephalus, *and Cylindrolaimus*), a plant-parasite *Tylenchorhynchus*, a fungivore *Aphelenchus*, and an omnivore *Acromadora* explained more variation at harvest.

The relative differences in indices values among treatments did not significantly change from fall harvest to the following spring, as evident by no significant interaction of treatment and season for any type of index value (Table 3). However, trophic diversity (N1) pooled over treatments significantly decreased over the winter, and was also consistently greater in *Bt* than the isoline with or without insecticide as evident by the significant treatment effect (Fig. 3, Table 3). Index values of maturity, proportion of fungivores and EI also decreased from fall harvest to spring but overall treatment means were similar statistically. Total abundance, proportions of non-fungivore trophic groups, and index values of CI and SI were not affected by the main effects of treatment or phenology.

Non-significant interaction effects indicated that there were no significant differences in the abundance and community structure of nematodes from pre-plant to two weeks after planting, except for the proportion of predaceous nematodes. This trophic group increased two weeks after the application of insecticide in the isoline plots but decreased in plots of *Bt* and isoline without insecticide (Fig. 4, Table 4).

3.2. Root decomposition

Nematode community indices all changed through the nine months of root decomposition, except for the proportion of predaceous nematodes, but month had no two-way interactions with treatment (Table 5). Pooled over months, MI values and proportion of predaceous nematodes in communities were consistently lower in non-*Bt* isoline plots with insecticide compared to other treatments (Fig. 5). EEI values were greater for the isoline plots with insecticide than *Bt* (Table 5). *Tylenchorynchus* was the only plant-parasitic nematode genus observed in litter. Therefore, PPI was not reported for litter.

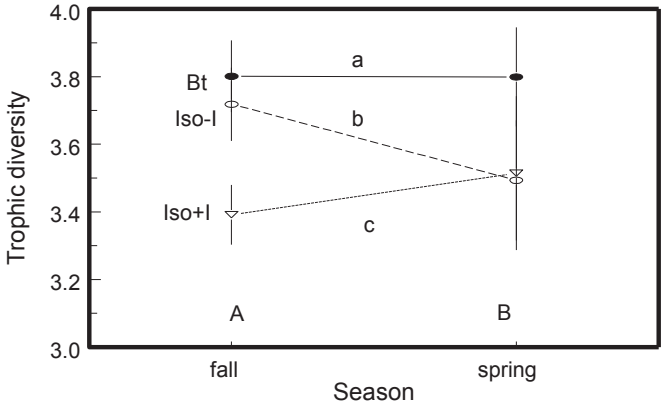

Fig. 3. Overwintering effect. Change from fall to spring of nematode trophic diversity in *Bt* hybrid (Bt), non-*Bt* isoline without insecticide (Iso − I), and non-*Bt* isoline with insecticide (Iso + I). Arithmetic means ± 1 SE are illustrated. Sample size was 36 after averaging subsamples (3 treatments × 2 times × 2 years × 3 replicates). Contrasting letters (lower case for treatment, upper case for time) represent statistical differences ($p \leq 0.05$).

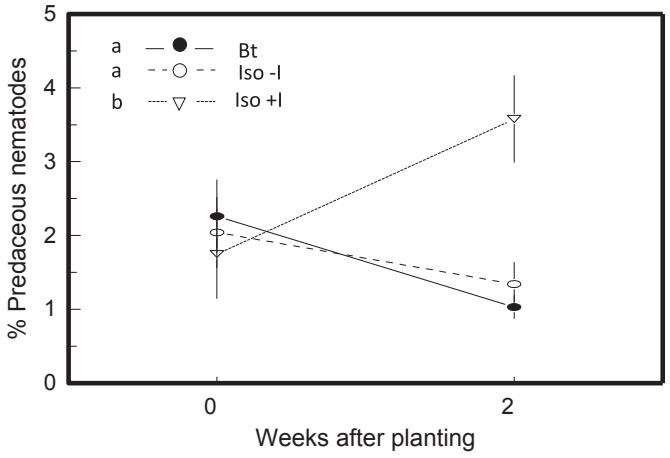

Fig. 4. Percentage of predators 0 and 2 weeks after planting. Arithmetic means ± 1 SE are illustrated for *Bt* hybrid (Bt), non-*Bt* isoline − insecticide (Iso − I), non-*Bt* isoline + insecticide (Iso + I) treatments. The insecticide tefluthrin was applied at planting in the Iso + I treatment. Sample size was 6 per treatment after averaging subsamples (2 times × 3 replicates). Contrasting letters represent statistical differences ($p \leq 0.05$) of the two way interaction between treatment and time.

Table 4
Two-way ANOVA on short-term effects of insecticide (0 and 2 weeks after planting) on nematode communities in soils with corn treatments (Bt, Isoline − insecticide, Isoline + insecticide). Sample size was 18 per treatment after averaging subsamples (3 treatments × 2 times × 3 replicates). Row and column of the Latin square design were treated as random variables.

Index	Treatment (df = 2)		Time (df = 1)		Treatment × Time (df = 2)	
	F-values	*P*-values	*F*-values	*P*-values	*F*-values	*P*-values
ln (density)[a]	0.08	0.9210	0.10	0.7590	0.03	0.9719
PPI[b]	3.92	0.0554	0.08	0.7771	2.07	0.1763
MI[b]	0.84	0.4613	0.09	0.7761	0.56	0.5860
Plant-Parasites[c]	0.28	0.7590	0.03	0.8748	1.24	0.3294
Fungivores[c]	0.31	0.7393	2.51	0.1440	0.21	0.8132
Omnivores[c]	1.49	0.2723	2.54	0.1422	1.58	0.2527
Bacterivore[c]	0.57	0.5810	0.33	0.5788	0.59	0.5736
Predators[c]	2.69	0.1164	0.10	0.7579	5.90	0.0203
N1trophic[d]	1.61	0.2478	0.70	0.4235	0.73	0.5079
CI[e]	0.60	0.5672	0.03	0.8695	0.77	0.4887
EI[e]	0.99	0.4057	0.82	0.3862	0.13	0.8769
SI[e]	0.51	0.6159	0.40	0.5436	0.08	0.9214

[a] Transformed as ln (x + 0.1).
[b] Successional maturity indices of nematode communities: PPI (plant-parasitic nematodes cp 2 to cp5), MI (free-living nematodes cp1−cp5), Maturity indices are weighted means computed as Σ [CP-value (i)*f(i)]/[total numbers of nematodes] where (i) is the individual taxon and f(i) is the frequency of the taxon in a sample (Bongers, 1990).
[c] Trophic groups of nematodes, transformed as arcsine of the square root.
[d] Trophic diversity: exp − ∑ [P_i (ln P_i)] where P_i is the proportion of group (trophic level) i in the total nematode community and reflects food web complexity.
[e] Channel index (CI), enrichment index (EI), and structural index (SI) (Ferris et al., 2001).

4. Discussion

There are several possible routes of exposure by which rootworm-active *Bt* corn could affect soil microorganisms. Herbivorous nematodes can be exposed directly by consuming Cry proteins expressed in living roots. Also, toxin proteins exuded by root senescence after anthesis or released from decomposing plant residue after harvest can enter the soil food web as a food substrate in the diet of herbivorous nematodes and saprophytic microbes. Saprophytic fungi that feed on these substrates are the food sources of fungivorous nematodes. Bacteria, which also decompose the substrates, are food sources of bacterivorous nematodes. In turn, bacterivores are the food sources of predaceous nematodes.

Our results support the hypothesis that *Bt* corn does not affect adversely non-target soil nematodes in the rhizosphere and decaying roots. There is evidence that rhizosphere soil in *Bt* corn may contain more complex and successionally mature nematode communities than those treated with conventional insecticide, and this effect continued over winter to the following spring prior to planting. Our findings support those of Höss et al. (2011) who found the nematode communities examined at the genus level to be distinct among the *Bt* (Mon88017) and isoline, i.e., cultivar-specific community structures. However, crop phenology had a greater effect on nematode community composition than corn treatment in our study. Moreover, it is documented that nematode composition varies among corn varieties, whether or not they contain *Bt* proteins (Griffiths et al., 2005, 2007).

MI is a measure of disturbance, with smaller values being indicative of a more disturbed environment than larger values. Values of MI were similar in *Bt* and non-*Bt* isoline and both greater than non-*Bt* isoline with insecticide. This finding contrasts that of Höss et al. (2011) who suggest that MI values are less in Cry3Bb than isoline at anthesis but not at planting or harvesting. In contrast, Höss et al. (2011) attributed contrasting MI values to greater and less relative abundances of *Rhabditis* (CP1) and *Alaimus* (CP4), respectively, in *Bt* compared to the near-isogenic cultivar. However, the lower MI values could not be attributed unequivocally to the *Bt*-treatment.

Parallel to ecological succession indices, we found SI equal in *Bt* and non-*Bt* isoline without insecticide but both greater than non-*Bt* isoline with insecticide. SI is an indicator of food web state affected by stress or disturbance. EI of the non-*Bt* isolate with or without insecticides was greater than *Bt*. EI is a measure of opportunistic bacterivore and fungivore nematodes. Values of EI were smaller in our study than Höss et al. (2011) placing our communities into quadrant C (lower right) rather than quadrant B (upper right) of the structure and enrichment conditions of the soil food web (Ferris et al., 2001). Relatively high EI and SI values reflect the N-enriched, low to moderately disturbed conditions that are typical for perennial crop agriculture (Ferris et al., 2001). Relatively low CI values, such as those obtained in this study, reflect decomposition channels of the soil food web that are mainly dominated by bacterial than fungal decomposition (Ferris et al., 2001). Nonetheless, these values were the same for all corn treatments.

With one exception, our results support Al-Deeb et al. (2003) who observed no significant effects of Cry3Bb on total abundance,

Table 5
Two-way repeated measures ANOVA on nematode communities in decaying roots of corn treatments (*Bt*, non-*Bt* Isoline − insecticide, non-*Bt* Isoline + insecticide). Time of decomposition (1, 2, 3, 8, and 9 months) was treated as a repeated measure, adjusted for within-subject correlation using an autoregressive structure. Sample size was 90 per treatment after averaging subsamples (3 treatments × 5 months × 2 years × 3 replicates). Year, row and column of the Latin square design were treated as random variables.

Index	Treatment (df = 2)		Months (df = 4)		Treatment × Months (df = 8)	
	F-values	*P*-values	*F*-values	*P*-values	*F*-values	*P*-values
ln (density)[a]	0.27	0.7658	37.25	<0.0001	0.93	0.5004
MI[b]	5.66	0.0052	25.19	<0.0001	1.53	0.1609
Plant-parasites[c]	1.13	0.3295	3.36	0.0141	1.14	0.3463
Fungivores[c]	0.93	0.3983	4.21	0.004	0.52	0.8408
Omnivores[c]	0.32	0.7255	11.50	<0.0001	1.19	0.3158
Bacterivores[c]	0.21	0.8116	4.20	0.0041	0.79	0.6166
Predators[c]	0.64	0.0327	0.84	0.5014	1.38	0.2213
N1trophic[d]	0.64	0.5288	5.25	0.0009	1.55	0.1554
CI[e]	0.63	0.5337	6.69	0.0001	0.70	0.6883
EI[e]	11.32	<0.0001	38.78	<0.0001	0.97	0.4681
SI[e]	2.59	0.0821	13.53	<0.0001	0.66	0.7251

[a] Transformed as ln (x + 0.1).
[b] Successional maturity indices of nematode communities: weighted means computed as Σ [CP-value (i)*f(i)]/[total numbers of free-living nematodes] where (i) is the individual taxon and f(i) is the frequency of free-living taxa in a sample (Bongers, 1990).
[c] Trophic groups of nematodes, transformed as arcsine of the square root.
[d] Trophic diversity: exp − ∑ [P_i (ln P_i)] where P_i is the proportion of group (trophic level) i in the total nematode community and reflects food web complexity.
[e] Channel index (CI), enrichment index (EI), and structural index (SI) (Ferris et al., 2001).

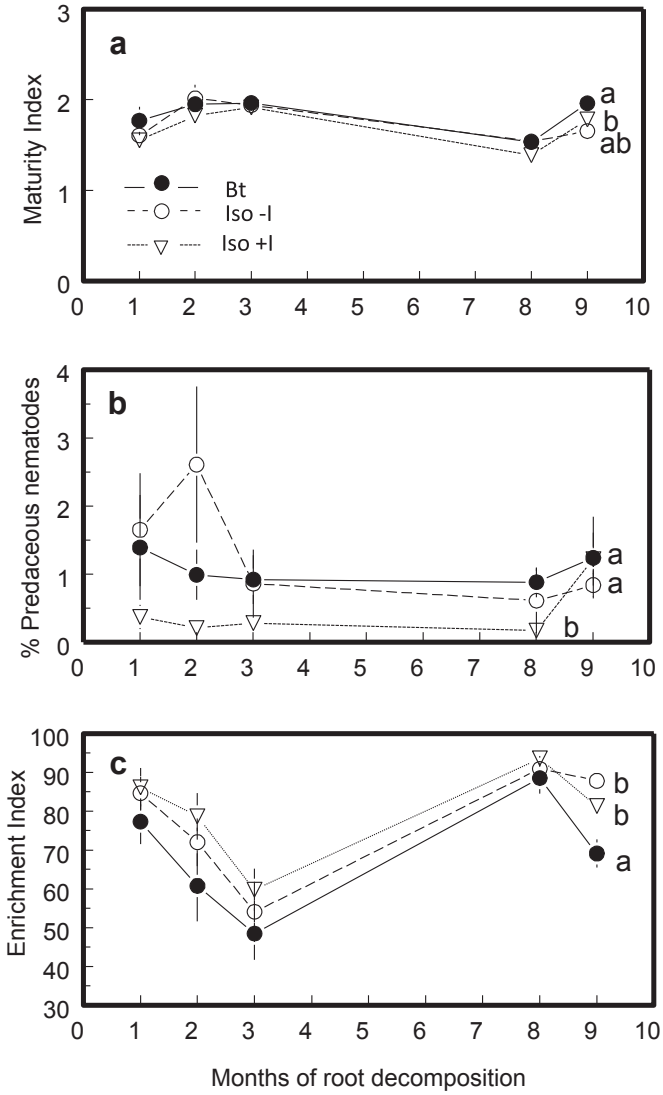

Fig. 5. Decomposition of corn roots. Corn treatment effects on a) maturity index of free-living nematodes and b) percentage of predators, and c) enrichment index at 1, 2, 3, 8 and 9 months of decomposition. Arithmetic means ± 1 SE are illustrated for *Bt* hybrid (Bt), non-*Bt* isoline − insecticide (Iso − I), non-*Bt* isoline + insecticide (Iso + I). Sample size was 90 after averaging subsamples (3 treatments × 5 months × 2 years × 3 replicates). Contrasting letters represent statistical differences ($p \leq 0.05$).

number of genera, or proportion of trophic groups. In our study, the relative abundance of fungivorous nematodes was greater in *Bt* than non-*Bt* isoline hybrids. Manachini and Lozzia (2002) also noted a relative abundance of fungivorous nematodes dominating in soil from *Bt* corn fields (Event 176, Novartis). However, this occurred at one but not a second location.

Results of this study support the mounting evidence that *Bt* corn is pest-specific and does not have any statistically negative effects on non-target soil fauna (Saxena and Stotzky, 2000; Carter et al., 2004). Not only are mites and collembolans unaffected but *Bt* corn does not affect other non-target soil inhabitants including beetles, microorganisms, protozoa, other microarthropods, nematodes or earthworms (Pilcher et al., 1997; Lozzia et al., 1998; Dutton et al., 2002; Al-Deeb et al., 2003; Candolfi et al., 2004; Devare et al., 2007; Rose and Dively, 2008; Lawhorn et al., 2009).

It was unexpected that the effects observed during the season would carry overwinter into the next season. The half-life of Cry3Bb1 protein in decomposing MON863 corn leaf, stalk and root residue is less than 6 days (Prihoda and Coats, 2008). At the end of 25 days, less than 1% of the *Bt* Cry3Bb1 protein remained in leaf, stalk and root tissues. There was a trend of increasing half-life of *Bt* Cry3Bb1 protein in MON863 corn residue in microcosms with macro-decomposers (earthworms, isopods, springtails) present as compared to the treatment containing MON863 corn only (Prihoda and Coats, 2008).

Bt corn was developed as a substitute of traditional chemical insecticide in corn rootworm management, so it is important to assess the relative ecotoxicological risk of both control strategies. The addition of the conventional insecticide clearly had a tendency to have greater effects on non-target nematodes than coleopteran-active *Bt* corn. Tefluthrin is a synthetic pyrethroid which has been commonly used as a soil insecticide for broad spectrum control of soil insects. The application of this insecticide decreased abundance of nematodes at higher positions in the food chain and shifted ecological succession back to earlier stages compared to *Bt* or the isoline without insecticide.

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