1 The effects of the presence of *Bt*-transgenic oilseed rape in wild mustard populations
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3 on the rhizosphere nematode and microbial communities
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Abstract

The adventitious presence of transgenic crops in wild plant populations is of ecological and regulatory concern. In this context, their effects on non-target, below-ground organisms are not well understood. Here, we introduced, at various frequencies, *Bt*-transgenic oilseed rape (OSR, *Brassica napus*) into wild mustard (*B. juncea*) populations in the presence and absence of the target herbivore (*Plutella xylostella*). The impacts on soil nematode and microbial communities were assessed in this system. There were no significant changes on the number of nematode genera and abundance in proportions of OSR with mustard. Nonetheless, the Shannon-Wiener and Pielou evenness index was lowest in plant stands containing 50% of *Bt*-transgenic OSR. Among treatments, there was no significant variation for culturable soil microbes. There was a positive association between foliar herbivory and the abundance of plant parasitic (PP) and cp-3 nematodes, whereas there was no association between herbivory and soil microbial populations. There was no direct effects of the presence of *Bt*-transgenic OSR in wild mustard populations on the rhizosphere nematode and microbial communities, whereas its indirect effects via aboveground herbivory might be important to consider for biosafety assessments.

Key words: Adventitious presence; *Brassica juncea*; *Bt*-transgenic oilseed rape; Microbial community; Soil nematodes.

1. Introduction

As the global cultivation area of genetically modified (GM) crops increases (James, 2012), the risk of persistence of transgenic crops in native ecosystems are being assessed (Darmency, 1994; Ellstrand et al., 2013; Liu et al., 2013). GM plants might have a competitive advantage when fitness-related transgenes (e.g., those conferring insect resistance) are expressed. These plants and/or their transgenes could persist in natural ecosystems as landraces, feral plants and volunteers through gene flow and introgression via pollen dispersal (Ellstrand et al., 2013; Liu et al., 2013; Stewart et al., 2003; Warwick et al., 2008) or seed movement (D'Hertefeldt et al., 2008; Reuter et al., 2008; van Heerwaarden et al., 2012).

If GM plants persist in the environment, effects on non-target organisms (NTO), including those that exist underground in the rhizosphere, should be assessed (Knox et al., 2007; Li and Liu, 2013; Yang et al., 2014). Proteins (e.g. *Bacillus thuringiensis* proteins) from transgenic plants can enter the soil through plant litter and root exudates (Knox et al., 2007; Li and Liu, 2013). Once *Bt* proteins are released in the soil, mineral and organic soil particles can bind *Bt* toxin proteins (Saxena and Stotzky, 2001; Tapp et al., 1995), but the *Bt* proteins do not persist or accumulate in soil (Badea, 2010; Icoz and Stotzky, 2008a). *Bt*-crops are generally assumed having few-to-no toxic effects on earthworms, nematodes, woodlice, collembolans, mites and protozoa (Icoz and Stotzky, 2008b). *Bt*-transgenic cotton was shown to have no negative impacts on nematode communities compared to non-transgenic cotton in field studies conducted over two- (Yang et al., 2014), or ten-years (Li and Liu, 2013).

1 58 However, some studies have reported the negative effects of *Bt* proteins on soil
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3 59 invertebrates, including nematodes (Griffiths et al., 2005; Hoss et al., 2008; Hoss et
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6 60 al., 2014; Wei et al., 2003), microbes (Turrini et al., 2004; Xue et al., 2005) and
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9 61 earthworms (Lang et al., 2006; Zwahlen et al., 2003). Most of these studies compared
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11 62 transgenic crops and their non-transgenic isogenic counterparts in the greenhouse
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14 63 and/or field. It is not clear how transgenic crops that persist in wild habitats might
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17 64 affect soil invertebrates; mixtures of plant species could affect community
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20 65 composition of soil organisms (e.g. Wardle et al., 2003).
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22 66 In addition, aboveground herbivores can indirectly affect belowground
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25 67 organisms by damaging the plant and affecting the quantity and quality of primary
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28 68 and secondary metabolites in the belowground biomass (Bardgett and Wardle, 2003;
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31 69 Frost and Hunter, 2008; Kaplan et al., 2009; Wardle et al., 2004). Above-ground
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34 70 herbivory can have both positive and negative effects on below-ground organisms,
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37 71 which vary with other factors (Bardgett and Wardle, 2003; Bezemer and van Dam,
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39 72 2005; De Deyn et al., 2007; Wardle et al., 2004), such as insect-resistant traits of crop
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42 73 cultivars (Huang et al., 2012).
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44 74 In this study, we simulated the persistence of a transgenic plant in a wild
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47 75 congener using increased percentages of *Bt*-transgenic oilseed rape (*Brassica napus*,
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50 76 OSR) in wild brown mustard (*Brassica juncea*) stands to assess the effects of this
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53 77 persistence on nematode and soil microbial community. The global cultivation area of
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56 78 transgenic OSR ranks fourth among all GM crop species (James, 2012). Wild brown
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59 79 mustard is a weedy plant in agricultural systems in China and is closely related to
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OSR. Both species are polyploids. Both species can easily intercross and produce viable progeny (Frello et al., 1995; Liu et al., 2010), and the population dynamics of wild brown mustard, therefore, could potentially be affected by *Bt* transgene introgression. In the present study we aim to investigate 1) whether the simulated invasion process of *Bt* OSR into wild mustard populations affects the soil nematode- and microbial communities; 2) whether aboveground herbivory influences soil organisms via plant responses.

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2. Materials and methods

2.1. Plants

Seeds of wild brown mustard (*Brassica juncea*, $2n = 36$, AABB) from a local field collection (Nanjing, China) were provided by Prof. S. Qiang, Nanjing Agricultural University (NAU). Transgenic oilseed rape (OSR, *Brassica napus* cv. Westar, $2n = 38$, AACC) was transformed with a pSAM12 plasmid containing genetically linked *gfp* (encoding a green fluorescent protein) and *Bt Cry1Ac* cassettes (GT) that are regulated by independent CaMV 35S promoters (Halfhill et al., 2001). The third generation of progeny of a single transgenic event (GT1) contained a single transgene insertion or multiple copies at a single locus (Halfhill et al., 2001). The Cry 1Ac protein of *Bt B. napus* plants of this generation was measured by ELISA and the protein content was $4.47 \mu\text{g g}^{-1}$ fresh weight on average at flowering.

2.2. Persistence experiments

Seeds of *Bt* OSR and wild brown mustard were sown in Jiffy-7 pots in the greenhouse

(22 °C under natural light) on April 16, 2012, and seedlings were transplanted to cages (2 m width × 2 m length × 2 m height, protected by a 0.5 mm mesh nylon net) after two weeks. Thirty cages were deployed in five rows and six columns in a field, with 2 m distance between cages, which provided adequate light for plant growth in cages. To simulate the invasion process of *Bt*-transgenic plants into a wild population, the two types of plants (OSR and mustard) were cultivated in pure stands or in five different mixtures in a replacement series (Fig. 1): 0% (P_0), 25% (P_{25}), 50% (P_{50}), 75% (P_{75}), and 100% (P_{100}) of OSR plants. In mixed stands, individuals among species were alternated: in P_{50} , and one beside two or three in the cages of P_{25} and P_{75} , respectively. Each cage included 36 plants arranged in six rows and six columns. Cages of the five different mixtures were placed randomly in a column. Thirty cages were deployed in three blocks. One-half of the cages in each block was infested with insects, whereas the other half was not infested (control). Two second-instar larvae of *Plutella xylostella* L. were applied per plant on May 19. No insecticide and fertilizer was applied during the experiment. Plants were watered and hand-weeded as needed.

2.3. Soil samples

Soil samples were collected in plant rhizospheres at depths of 0-10 cm on three dates: May 30th, July 4th and August 14th 2012. Three 100 cm³ soil samples were taken per cage. These soil samples were evenly mixed and transported back to the laboratory where nematodes were extracted. Microbial communities were assayed only for soil samples taken on July 4th because the *Bt* concentration was highest at this date when plants flowered (Lei et al., 2011). Soil characteristics, including pH, temperature and

1 124 electrical conductivity, were measured using TRIME-PICO-IPH TDR (Germany).

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3 125 Organic carbon in air-dried soil samples was measured according to the standard

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6 126 methods recommended by the Chinese Society of Soil Science (Lu, 2000). 2.4.

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9 127 *Nematodes*

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11 128 Nematodes from 100 cm³ soil samples were isolated within 48 h using the modified

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14 129 Baermann method (Ingham, 1994). Suspensions of nematodes were collected and

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17 130 concentrated to 2 ml and then preserved at 4°C with 4% (v/v) formaldehyde, then

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20 131 identified using common classification methods (Goodey, 1963; Liu, 2004; Yin,

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22 132 1998). We identified nematodes to genus under 200× magnification with an inverted

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25 133 compound micro-scope.

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28 134 *2.4.1. Nematode biodiversity*

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34 136 Shannon-Wiener index (Shannon, 1948): $H' = -\sum P_i \ln P_i$, where P_i is the proportion of

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37 137 individuals in the i -th taxon; the Pielou evenness index (Pielou, 1975): $E = H' / \ln S$,

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40 138 where S indicates the richness (number) of taxa; and the Simpson dominance index

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42 139 (Simpson, 1949): $J = 1 / \sum P_i^2$ were all calculated during the study.

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45 140 *2.4.2. Nematode functional guilds*

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47 141 Nematodes were also classified into four feeding types: bacterivores (Ba), fungivores

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50 142 (Fu), plant parasites (PP) and omnivores-predators (Op) (Pen-Mouratov et al., 2004;

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53 143 Steinberger and Loboda, 1991; Yeates et al., 1993). Nematode taxa were rated along a

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56 144 colonizer-persister (c-p) scale of 1-5 according to Bongers and Ferris (1998). Maturity

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59 145 index (MI) and plant parasites index (PPI) were calculated: $MI = \sum v(i) / f(i)$, $PPI = \sum v(i)$

$\times f(i)$; where $v(i)$ is the $c-p$ value of the i -th taxon; $f(i)$ is the frequency of taxon i in a sample (Bongers, 1990; Bongers and Bongers, 1998). The MI and PPI indices were calculated using a $c-p$ value that ranges from colonizer ($c-p = 1$ or 2) to persister ($c-p = 5$). Nematode channel ratio (NCR) (Bongers and Bongers, 1998) and Wasilewska index (WI) (Wasilewska, 1998) were calculated: $NCR = N_{Ba} / (N_{Ba} + N_{Fu})$, $WI = (N_{Ba} + N_{Fu}) / N_H$, where N_{Ba} is the abundance of bacterial-feeders (Ba), N_{Fu} , the abundance of fungal-feeders (Fu), N_H , the abundance of plant parasites (PP). The enrichment index (EI) and structure index (SI) were calculated according to Ferris *et al.* (2001).

2.5. Microbial community

2.5.1. Plate counts of microbial colonies

Microbes were cultured from the soils sampled on July 4th. Ten grams of soil was diluted and the soil suspensions were each distributed in duplicate onto three selective media: beef extract peptone medium for bacteria, Czapek medium for fungi, and Gause I medium for actinobacteria. Soil samples were serially diluted to 10^{-2} and 10^{-3} suspensions for bacteria and actinobacteria, and to 10^{-1} and 10^{-2} suspensions for fungi. The plates were incubated at 35-37 °C and colonies were tallied each day for three days. The sum of all colonies for each type of microbe was used for statistical analysis.

2.5.2 Eco-BIOLOG profiles

Soil samples were serially diluted to a 10^{-3} suspension in sterile 0.1 mol L⁻¹ phosphate buffer (adjusted to pH 7.0) for inoculation to *Eco*-Biolog plates. Each *Eco*-Biolog plate well was inoculated with 150 µL suspension at 30°C in the dark and scanned every 24 h at 590 nm for 1 wk. The data were collected by Microlog Release software

(ML3402, Microlog, USA). The 72 h absorbance values were used to calculate diversity indices and PCA (Zhang et al., 2008). Community-level physiological profiles (CLPP) diversity was calculated by the Shannon index: $H' = -\sum P_i \ln P_i$, where P_i is the proportion of color development of the i th well over total color development of all wells of a plate, and evenness index was calculated as $E = H' / \ln S$, where S represents the number of wells with color development; the calculation of H' and E were based on 72 h incubation readings (Zhang et al., 2008).

2.6. Statistical analyses

The mean values of each variety measured per cage were used for statistical analyses. Data distributions were tested for normality using the Shapiro-Wilk test. Bartlett's test was used to test for homogeneity of variance. A mixed ANOVA ($Y \sim I \times P \times D + \text{Error (block)}$) was employed to test the effects of insect infestation (I), planting patterns (P), and sampling dates (D) on nematode communities. A mixed ANOVA ($Y \sim I \times P + \text{Error (block)}$) was employed to test the effects of insect infestation (I), and planting patterns (P) on microbial communities, including the colony counting and diversity indices of microbes. Tukey's HSD test was used for multiple comparisons among the invasion process for all measured varieties. A contrast test was used to test the difference between pure stands of wild mustard and other stands. PCA was used to characterize community-level profiles of microbes. All statistical analyses were conducted using R software (R Development Core Team 2008).

3. Results

3.1. Nematode communities

Twenty-two families and thirty-eight genera of soil nematodes were identified during our study (Table 1). *Aphelenchus*, *Acrobeloides* and *Aphelenchoides* were the dominant genera, accounting for 37.6%, 12.6% and 9.3% of total assayed nematodes, respectively. The nematode abundance in the field was 1.9×10^4 , 1.1×10^4 and 6.0×10^3 per 100 cm³ soil on May 30th, Jul. 4th and Aug. 14th, respectively. The average percentage considering all treatments of bacterivores (Ba), fungivores (Fu), plant parasites (PP) and omnivores-predators (Op) nematodes was 33.7%, 47.5%, 14.4% and 4.40%, respectively.

The abundance of plant parasites (PP) and cp-3 nematodes was significantly increased in infested treatments (Table 2, Fig. 1). All other nematode parameters were not affected by herbivore treatment. All community indices and life characteristics of nematodes were not significantly different between stands with and without insect infestation (Table 2).

The simulated invasion of *Bt*-transgenic *B. napus* in wild *B. juncea* did not significantly affect the species number and abundance of nematodes (Table 2, Fig. 1). It had significant effect on the J (Simpson), H' (Shannon), evenness (E) and SI indices (Table 2), and the significant difference among the five planting patterns was found at the Aug. 14th sampling date, but there were no effects at other sampling dates (Fig. 2). The minimum value of indices H' and E, and the maximum dominance index J appeared in the mixed stands (P₅₀) with 50% of *Bt*-transgenic OSR post-harvest (Fig. 2). These indices of nematodes in the mixed patterns of *Bt*-transgenic oilseed rape and

wild mustard was not significantly different compared to that observed in pure stands (P>0.05; Contrast test, data not shown).

The nematode abundance decreased from May 30th to Aug. 14th ($F_{2,84}=36.6$, $P<0.001$), although the number of genera was not different among the three sampling dates (Table 1 and 2, Fig. 2). Between May and August sampling, the number of fungivorous nematodes decreased from 59.3% to 35.7% ($F_{2,84}=41.1$, $P<0.001$), while the number of bacterial feeders (from 29.9% to 41.4%) and plant parasites (from 6.7% to 19.6%) increased ($F_{2,84}=6.80$, $P=0.002$ for bacterial feeders; $F_{2,84}=21.7$, $P<0.001$ for plant parasites); the number of *cp-2* scale nematodes decreased from 85.7% to 59.2% ($F_{2,84}=19.8$, $P<0.001$), while the number of *cp-4* increased from 14.5% to 87.7% ($F_{2,84}=7.26$, $P=0.002$); the indices, J, H', E and NCR increased ($F_{2,84}=11.5$, $P<0.001$ for J; $F_{2,84}=8.7$, $P<0.001$ for H'; $F_{2,84}=15.9$, $P<0.001$ for E; $F_{2,84}=16.5$, $P<0.001$ for NCR). Other nematode community parameters did not significantly change with time.

The graphic presentation of faunal analysis showed that five invasion stages were mapped in quadrat D and they clustered together (Fig. 3). A lower structure index (SI) was observed in plant populations without *Bt* OSR (P_0) in the no-insect treatment compared with other population compositions (Fig. 3), whereby the SI was 16.4, 26.0, 26.67, 23.16 and 26.15 for P_0 , P_{25} , P_{50} , P_{75} and P_{100} , respectively. The enrichment index (EI) was 36.07, 40.42, 40.37, 38.2 and 38.13 for P_0 , P_{25} , P_{50} , P_{75} and P_{100} , respectively. In infested treatments, P_{100} was separated from the other percentages, with a higher SI (Fig. 3), and the SI was 23.4, 16.6, 14.57, 21.1 and

33.46 for P₀, P₂₅, P₅₀, P₇₅ and P₁₀₀, respectively. The EI was 37.87, 40.38, 39.20, 37.84 and 39.33 for P₀, P₂₅, P₅₀, P₇₅ and P₁₀₀, respectively.

3.2. Microbial community

Insect infestation in pure or mixed stands of *Bt*-transgenic *B. napus* and wild *B. juncea* did not affect the colony number of culturable microbes in the soil (Table 3). The number of culturable microbes was also not significantly different among the five proportions of plant composition (Table 3). However, contrast analysis showed that the number of fungi and actinomyce colonies in pure stand of wild mustard (P₀) was lower than that in other stands ($t=2.36$, $P=0.03$ for fungi; $t=2.11$, $P=0.04$ for actinobacteria), but not for the number of bacterial colonies and total microbe colonies (bacterial + fungi + actinobacteria) ($P>0.05$, contrast test) (Fig. 4). The culturable microbes were fewest in the P₅₀ stands (Fig. 4). There was no significant interaction between infestation and planting patterns except for the colony number of bacteria (Table 3). A contrast test showed that bacterial colony number in pure stands of *B. juncea* (P₀) was higher than in other plant compositions (P₂₅+P₅₀+P₁₀₀) ($t=3.63$, $P=0.005$) only in the absence of insects.

Insect infestation treatments did not affect the microbial community diversity indices, and Shannon H' and E, and the diversity indices of soil microbes among the five invasion stages (Table 3). There were no interactions between infestation and planting patterns for the two diversity indices (Table 3). The PCA plot of the CLPP explained 39.0% of total variance, with PC1 accounting for 22.0%. There were no differences of PCA variance between herbivore treatments, suggesting that the

herbivore treatment did not affect the utilization of the carbon sources by soil microbes (S1_Fig).

3.3. Soil characteristics

No significant differences were observed for soil pH, temperature, electrical conductivity and organic carbon among five percentages of plant composition. The soil temperature on Aug. 14 was higher than that on May 30 and Jul. 04, but there was no significant differences observed among the three sampling dates for other measured characteristics (S2_Fig).

4. Discussion

4.1. Effects of herbivory on soil organisms via plant responses

Above-ground herbivory can influence rhizosphere ecology, including food webs, by altering root exudation and carbon allocation by changing leaf litter quality and quantity (Bardgett et al., 1998; Huang et al., 2012). The indirect effect of above-ground herbivores on below-ground organisms varied with other factors, for example brown planthopper (*Nilaparvata lugens*) decreased nematode abundance and microbial biomass when feeding on susceptible rice, but an opposite response was found when feeding on insect-resistant rice (Huang et al. 2012). Here, we found that foliar herbivory (*Plutella xylostella*) treatments had an effect wherein there were increased abundances of plant parasites (PP) nematodes and *cp-3* taxa. This is because *P. xylostella* might induce plants to alter primary and secondary metabolites production that affected the abundance of plant-parasitic nematodes. The *cp-3*

nematodes are characterized by long generation times, high sensitivity to adverse
 conditions, and include fungivores, bacterivores and carnivores (Ferris et al., 2001).
 Worldwide, plant parasitic nematodes cause considerable crop damage, and thus, it is
 important to consider them here (Lingaraju et al., 2012). *Helicotylenchus*,
Pratylenchus, *Filenchus*, *Tylenchus* were the most dominant genera of plant parasitic
 nematodes in this study, which was consistent with findings in cotton fields in China
 (Li and Liu, 2013).

In the present study, there were no direct effects of aboveground foliar
 herbivory on microbial communities. Brown planthopper presence affected microbial
 biomass and nematode abundance (Huang et al., 2012). Root-feeding nematodes
 were more abundant in soil when grasses were defoliated because of high biomass
 allocation to roots, which resulted in the increased rates of root exudation (Ingham
 and Detling, 1986). Increases in nematode infestation in defoliated plants can enhance
 the leakage of carbon from plant roots into the rhizosphere (Yeates et al., 1998),
 which could increase soil microbial biomass. However, foliar herbivory did not affect
 the microbial community in our study, which could explain why Ba, Fu and OP
 nematodes were not affected by aboveground herbivory. Defoliation of grassland
 species increased the number of culturable bacteria but had no effects on the fungal
 community (Mawdsley and Bardgett, 1997). It was suggested there that defoliation
 promotes fast cycles dominated by high quality root exudates, which favor bacterial
 growth. In agroecosystems, grasshopper-damaged maize had increased soil microbial
 biomass in no-till systems, but there were no significant effects in tilled fields

(Holland et al., 1996).

4.2. Microbial community dynamics during GM crop invasion

There was no significant difference in microbial communities found during the simulated invasion process of *Bt* OSR into wild mustard habitats. Previous studies reported similar results in soil microbe communities (Baumgarte and Tebbe, 2005; Miethling-Graff et al., 2010; Rui et al., 2005). No negative effects of *Bt*-cotton cultivation on the functional diversity of microbial communities in the soil (Shen et al., 2006). *Bt*-transgenic maize MON863 expressing cry3B1 toxin had no adverse effect on saprophytic microbial communities (Lawhorn et al., 2009). The differences was also observed of microbial communities between transgenic and non-transgenic *B. napus*; however, the differences were temporary and not observed in the next field season (Dunfield and Germida, 2003). In general, the effects of *Bt*-crops on microbial communities were transient and not related to the presence of *Cry* proteins (Icoz and Stotzky, 2008b). Since the culturable microbes represent only one segment of the soil microbial community, it under-estimates the entirety of the community, Nonetheless, our study provides more information of the effects of GM plants on culturable microbes and included the Biolog method to assess soil microbial structure and function (Zhang et al., 2008).

The contrast analysis (P_0 vs. $P_{25}+P_{50}+P_{100}$) in the present study showed that the number of fungi and actinobacteria colonies were higher after the invasion of *Bt* OSR, and their maximum abundance appeared in the mixed stands containing 50% *Bt* OSR plants wherein we observed high levels of plant competition. In the absence of

aboveground herbivory, the invasion of *Bt* OSR apparently decreased culturable bacteria, but no influence was found in the presence of herbivory. Taken together, these results suggest that the microbial communities were affected not only by plant species and characteristics, but also by aboveground herbivory. Small changes in genetics and biology can be important in this regard. For example, both oilseed rape variety and growth season induced the variation in the microbial community (Dunfield and Germida, 2003). Thus, the invasion of *Bt*-GM crops could indirectly affect microbial communities through the alteration of plant species composition and herbivory intensity, although GM crops have not been shown to have any direct negative impacts in this regard.

4.3. Nematode dynamics during the invasion process of GM crops

Many species of nematodes, and with high population densities, are commonly found in natural and agricultural sites. Thus, nematodes are often used as indicators of soil quality because of their participation in many functions at different levels of soil food webs. In this study, the plant growth season significantly affected nematode abundance, and the number of Ba and PP nematodes increased, whereas Fu nematodes decreased during the growing season to post-harvesting period. It is common that the structure of soil nematode communities are altered during cultivation, however, there seem to be no general patterns of community change (Li and Liu, 2013; Yang et al., 2014). Moreover, the seasonal effects were more significant on soil nematodes than that of aboveground herbivory and *Bt* OSR invasion.

344 To our knowledge, this is the first study to examine nematode dynamics in a
 345 simulated persistence of *Bt* transgenic crops within a weedy congener population.
 346 Previous studies on nematodes were mainly done by comparing the effects of
 347 transgenic and isogenic non-transgenic crops. For example, Manachini *et al.* (2004)
 348 showed more mycophagous nematodes and less phytophagous nematodes in soil
 349 planted with *Bt* OSR than in soil planted with the respective non-*Bt* isoline. The
 350 negative effects of *Bt* endotoxin protein on soil nematodes was also found in other
 351 studies (e.g. Griffiths *et al.*, 2005; Wei *et al.*, 2003). There are some reports of
 352 coleopteran active-*Bt* (Cry3Bb1) having impacts on soil nematodes communities
 353 (Neher *et al.*, 2014). Laboratory studies have demonstrated Cry1Ab, Cry3Bb1,
 354 Cry2Ab2, Cry 5B, Cry6A, Cry14A and Cry21A have deleterious effects on
 355 bacterivorous nematodes (Hoss *et al.*, 2013; Hoss *et al.*, 2011; Wei *et al.*, 2003). In
 356 contrast, numerous reports showed no apparent adverse effect of *Bt* crops on the
 357 community of soil nematodes (e.g. Li and Liu, 2013; Mina, 2011; Yang *et al.*, 2014).
 358 For example, the cultivation of *Bt*-cotton containing Cry1Ac or Cry1Ab/Ac had no
 359 negative effects on the abundance of soil nematodes (Li and Liu, 2013; Yang *et al.*,
 360 2014). Here, we tested a Lepidopteran active-*Bt* (Cry1Ab) where there are no
 361 anticipated effects on nematodes in a simulated persistence of *Bt* OSR within a weedy
 362 plant population.

363 In our studies, diversity indices of soil nematodes were different in the
 364 simulated invasion process at the time of post-harvest. The diversity index of soil
 365 nematodes was lowest in stands with 50% of *Bt*-OSR and 50% of wild mustard plants,

where plants between OSR and mustard were intensely competitive against one another. Therefore, it is possible that plant competition moderated root exudates to the rhizosphere. Organisms residing in the rhizosphere are known to be regulated by root exudates, and plant residues are the major source of carbon in the soil (Baetz and Martinoia, 2014). We speculate that the observed differences in diversity indices resulted in part from interactions between plant species studied here and probably not because of the *Bt*-transgene. Thus, the quality and quantity of root exudates, and changes in the chemical composition might be the factors determining the interaction strength of plant and soil organisms. Plant species have been observed to alter the diversity of microbiota and nematode communities (Wardle et al., 2003). For instance, Li and Liu (2013) found significant differences in the abundance of soil nematodes in cotton fields containing different cultivars, thus genomic differences in plants will likely play a greater role than *Bt* transgene expression. Therefore, although *Bt* OSR had no negative impacts on soil nematodes, its invasion could indirectly affect the community composition of nematodes through altering interplant competition and insect infestation.

5. Conclusions

Since *Bt* proteins could be released into soil through pollen, root exudates and plant residues from *Bt*-GM crops, their non-target effects in soil have attracted increasing attention. In the present study, no *Bt* toxin proteins were detected in soil. The simulated invasion process of *Bt* OSR in wild mustard habitats had little impact on

soil nematodes and microbial communities, but aboveground herbivory, interplant competition and plant species affected certain nematodes and microbial communities. Aboveground herbivory could indirectly affect soil invertebrates via plant responses, which will increase the soil NTO risks of GM plants because *Bt*-GM plants could directly kill targeted insects and indirectly affect the abundance of non-target insects in ecosystems. Thus, although the *Bt* proteins had no direct influence on soil nematodes and microbial community, it is likely that GM plants invasion indirectly affected the community composition of nematodes and microbes through changing insect infestation and plant competition.

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Figure legends

Fig. 1. The map of the infestation experiment. Infestation, insect release of *Plutella xylostella*; CK, without insect release. Each pane indicates one cage. The simulated invasion process with five percentages: P₀, P₂₅, P₅₀, P₇₅ and P₁₀₀ of *Bt*-transgenic *Brassica napus* (OSR). “R” in the pane indicates one OSR plant; “W”, wild brown mustard (*Brassica juncea*).

Fig. 2. Nematode community characteristics against three sampling dates. Bars are the mean values ($\pm$ SEM) of abundance, J, H' and E in the simulated invasion process with five percentages (P₀, P₂₅, P₅₀, P₇₅ and P₁₀₀) of OSR (*Bt*-transgenic *Brassica napus*). Different letters above the bars indicate significant differences in that group, and “ns” signifies no significance by Tukey's test.

Fig. 3. Faunal analysis of nematode community. Nematode communities in the five simulated invasion stages (P₀, P₂₅, P₅₀, P₇₅ and P₁₀₀) were mapped in quadrat D. Points show means and standard errors for enrichment and structure indices. Without insects, signifies the nematode community from soil planted with oilseed rape without insect release; with insects, insect treatment using *Plutella xylostella*.

Fig. 4. Number of culturable microbes and the frequency of three microbe taxa in the soil. There was no significant difference of culturable microbes in the simulated invasion process with five percentages (P₀, P₂₅, P₅₀, P₇₅ and P₁₀₀) of OSR.

Table 1. The abundance (per 100 cm³ soil), family, genera, feeding type and *c-p* values of soil nematodes in the persistence of *Bt* transgenic OSR in wild mustard populations at three sampling dates, May, July and August.

Family	Genera	Feeding type ^a	<i>c-p</i> value ^b	May	Jul.	Aug.
Panagrolaimidae	<i>Panagrolaimus</i>	Ba	1	655	34	131
Rhabditidae	<i>Rhabditis</i>	Ba	1	202	374	247
	<i>Mesorhabditis</i>	Ba	1	168	146	78
	<i>Diploscapter</i>	Ba	1	0	3	2
	<i>Rhabditidae</i>	Ba	1	6	176	0
Plectidae	<i>Plectus</i>	Ba	2	3	7	7
Cephalobidae	<i>Acrobeloides</i>	Ba	2	2446	1259	786
	<i>Acrobelus</i>	Ba	2	47	24	9
	<i>Chiloplacus</i>	Ba	2	1020	454	497
	<i>Eucephalobus</i>	Ba	2	787	771	569
	<i>Cephalobus</i>	Ba	2	332	258	254
Teratocephalidae	<i>Teratocephalus</i>	Ba	3	6	20	13
Monhysteridae	<i>Prismatolaimus</i>	Ba	3	0	80	66
Cyatholaimidae	<i>Ethmolaimus</i>	Ba	3	0	10	26
Alaimidae	<i>Alaimus</i>	Ba	4	15	15	50
Aphelenchoididae	<i>Aphelenchoides</i>	Fu	2	2642	336	348
Aphelenchidae	<i>Aphelenchus</i>	Fu	2	8485	3324	1626
Diphtherophoridae	<i>Diphtherophora</i>	Fu	3	0	2	1
Leptonchidae	<i>Leptonchus</i>	Fu	4	0	24	9
Nothotylenchidae	<i>Nothotylenchus</i>	Fu	2	115	9	29
	<i>Boleodorus</i>	PP	2	117	445	80
Tylenchidae	<i>Tylenchus</i>	PP	2	51	0	6
	<i>Filenchus</i>	PP	2	205	125	103
	<i>Psilenchus</i>	PP	2	0	0	7
Belonolaimidae	<i>Tylenchorhynchus</i>	PP	3	9	27	83
Pratylenchidae	<i>Pratylenchus</i>	PP	3	265	933	297
	<i>Hirschmanniella</i>	PP	3	3	6	0
Hoplolaimidae	<i>Helicotylenchus</i>	PP	3	632	1198	553
Heteroderidae	<i>Heterodera</i>	PP	3	0	0	3
Seinurinae	<i>Seinura</i>	OP	2	45	0	0
Tripylidae	<i>Tobrilus</i>	OP	3	0	0	6
Dorylaimoidae	<i>Eudorylaimus</i>	OP	4	13	11	3
	<i>Dorylaimidae</i>	OP	5	191	203	19
	<i>Aporcelaimus</i>	OP	5	564	368	162
Discolaimus	<i>Discolaimus</i>	OP	5	0	16	8
Total				19019	10656	6076

Notes: ^a Feeding type: Ba, bacterivores; Fu, fungivores; OP, omnivores-predators; PP, plant parasites. ^b *c-p* value, colonizer persister.

Table 2. F-values of mixed ANOVA ($Y \sim I \times P \times D + \text{Error}(\text{Block})$) for nematode communities in the soil with five percentages of R (P) in the presence and absence of insect infestation (I) at three sampling dates (D).

	Infestation (I)	Percentage (P)	Date (D)	I×P	I×D	P×D	I×P×D
Genera	0.13	1.37	0.12	0.88	0.15	2.75*	0.31
Abundance	0.08	0.96	32.8***	0.56	0.6	0.81	0.74
PP	7.32**	0.44	22.4***	1.89	1.04	1.01	0.62
Ba	1.44	0.95	6.01**	0.33	0.36	0.43	0.48
OP	3.22	1.36	1.25	1.51	0.87	1.15	0.62
Fu	0.29	0.74	42.4***	0.73	0.23	1.24	1.46
<i>c-p</i> 1	0.52	0.89	0.86	0.71	0.06	0.71	0.54
<i>c-p</i> 2	2.22	0.35	19.1***	2.24	1.45	0.68	0.72
<i>c-p</i> 3	6.22*	0.23	15.4***	1.04	1.98	0.64	0.47
<i>c-p</i> 4	0.2	0.49	6.32**	1.28	0.78	0.51	0.67
<i>c-p</i> 5	2.65	1.62	1.31	1.44	0.87	1.23	0.66
J(Simpson)	0.17	2.58*	12.1***	2.43	2.4	1.3	0.6
H'	0.24	2.60*	9.93***	2.65*	0.8	2.35*	0.51
E	0.16	3.26*	16.7***	1.82	1.38	1.48	0.84
MI	0.14	1.83	2.07	1.04	0.44	1.06	0.63
PPI	0.27	1.02	2.12	1.58	2.42	0.78	0.52
NCR	0.24	1.07	14.9***	0.03	0.16	0.51	0.92
EI	0.03	0.7	5.19**	0.15	0.16	0.91	0.8
SI	0.81	2.82*	5.56**	3.65*	0.3	1.86	1.09

Ba, bacterivores; Fu, fungivores; OP, omnivores-predators; PP, plant parasites. *c-p* value, colonizer persister. J (Simpson), Simpson dominance index; H', Shannon-Wiener index; E, Pielou evenness index. MI, Maturity index; PPI, plant parasites index; NCR, Nematode channel ratio; EI, enrichment index; SI, structure index. *, indicates significant difference at $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 3. F-values of mixed ANOVA ($Y \sim I \times P + \text{Error}(\text{Block})$) for the number of culturable microbes (bacteria, fungi and actinobacteria) and diversity indices (Shannon H' and E) in soil with five percentages of OSR (P) in the presence and absence of insect infestation (I).

	Df	Culturable microbes			Diversity indices	
		Bacteria	Fungi	Actinobacteria	H'	E
Infestation (I)	1	0.19	1.01	0.02	0.02	0.95
Percentage	4	0.66	0.51	1.86	0.70	1.00
(P)						
$I \times P$	4	3.76*	0.39	1.33	1.63	1.13
Residuals	19					

Fig. 1

FIGURES

Fig. 2

Fig. 3

Fig. 4