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Harmful effects of mustard bio-fumigants on entomopathogenic nematodes

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ABSTRACT

Mustard (*Brassica* and *Sinapis* spp.) green manures tilled into the soil preceding potato crops act as bio-fumigants that are toxic to plant–parasitic nematodes, providing an alternative to synthetic soil fumigants. However, it is not known whether mustard green manures also kill beneficial entomopathogenic nematodes (EPNs) that contribute to the control of pest insects. We used sentinel insect prey (*Galleria mellonella* larvae) to measure EPN infectivity in Washington State (USA) potato fields that did or did not utilize mustard green manures. We found a trend toward lower rates of EPN infection in fields, where mustard green manures were applied, compared to those not receiving this cultural control method. In a series of bioassays we then tested whether the application of two mustard (*Brassica juncea*) cultivars, differing in glucosinolate levels, disrupted the abilities of a diverse group of EPN species to infect insect hosts. Mustard-exposure trials were conducted first in laboratory arenas where EPNs were exposed to mustard extracts suspended in water, and then in larger microcosms in the greenhouse where EPNs were exposed to green manure grown, chopped, and incorporated into field soil. In all trials we used *G. mellonella* larvae as hosts and included multiple EPN species in the genera *Steinernema* (*Steinernema carpocapsae*, *Steinernema feltiae*, *Steinernema glaseri*, and *Steinernema riobrave*) and *Heterorhabditis* (*Heterorhabditis bacteriophora*, *Heterorhabditis marelatus*, and *Heterorhabditis megidis*). In the laboratory, EPN infection rates were lower in arenas receiving mustard extracts than the control (water), and lower still when EPNs were exposed to extracts from plants with high versus low glucosinolate levels. Results were nearly identical when mustard foliage was soil-incorporated into greenhouse microcosms, except that the negative effects of mustards on EPNs developed more slowly in soil. Significantly, in arenas of both types one EPN species, *S. feltiae*, appeared to be relatively unaffected by mustard exposure. Together, our results suggest that the use of mustard bio-fumigants for the control of plant–parasitic nematodes has the potential to interfere with the biocontrol of insect pests using EPNs. Thus, it may be difficult to combine these two approaches in integrated pest management programs.

1. Introduction

Plant-parasitic nematodes pose a major pest threat to potato production in many parts of the world (Turner and Evans, 1998), including the irrigated potato-growing region in the Columbia Basin of Washington State and adjacent Oregon, USA (Ingham et al., 2005; Riga and Neilson, 2005). Particularly damaging in the Columbia Basin are Columbia root-knot nematode (*Meloidogyne chitwoodi* Golden, O'Bannon, Santo, and Finley), northern root-knot nematode (*Meloidogyne hapla* Chitwood), root lesion nematode (*Pratylenchus* spp.), and stubby root nematode (*Paratrichodorus* spp.) (Ingham et al., 1991, 2005; Riga and Neilson, 2005). Until recently, almost universally these harmful nematodes have been controlled using applications of broad-spectrum, synthetic soil fumigants (i.e., methyl bromide, metam sodium, and 1,3-dichloropropene). These synthetic soil fumigants are highly toxic to pests but also to many beneficial soil organisms (Schreiner et al., 2001; Cox, 2006). In addition, many of these conventional soil fumigants exhibit vertebrate toxicity and other damaging environmental effects (Cox, 2006). Together, these negative environmental and human health concerns have driven a search for more benign alternatives (Martin, 2003).

In recent years, potato growers in Washington have increasingly adopted the use of mustard (*Brassica* and *Sinapis* spp.) green manures, tilled into the soil preceding the planting of potato crops, as an alternative to synthetic soil fumigants (McGuire, 2003a). Mustard foliage and seeds contain glucosinolate compounds that upon hydrolysis produce isothiocyanates, which act as natural bio-fumigants (Brown and Morra, 1997). These bio-fumigant compounds suppress plant–parasitic nematodes (Riga et al., 2004; Zasada and Ferris, 2004), weeds (Brown and Morra,

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1995), pathogenic fungi (Kirkegaard et al., 1996), and other soil-borne pests. However, the relatively broad-spectrum activity of mustard bio-fumigants could lead to negative non-target impacts on beneficial soil flora and fauna, although this has not been previously investigated.

Included in the beneficial soil biota in regional potato fields are a diverse group of entomopathogenic nematodes (EPNs) in the genera *Steinernema* and *Heterorhabditis* (Liu and Berry, 1995; Berry et al., 1997). These nematodes enter host insects through natural openings (e.g. mouth, anus), then release a symbiotic bacterium that reproduces rapidly and kills the host (Boemare et al., 1996); the nematodes multiply by feeding on digested host tissues and these bacteria. Through conservation of endemic species, or application as bio-insecticides, EPNs have been shown to contribute to the biological control of the Colorado potato beetle (CPB), *Leptinotarsa decemlineata* Say (Berry et al., 1997; Armer et al., 2004), a key pest of potato in the Columbia Basin and many other potato-growing regions (Hare, 1990). Thus, any negative effects of soil-pest control tactics on these beneficial EPNs could disrupt biological control of pest insects in potato and other crops. Here, for the first time we report an examination of the non-target impacts of mustard bio-fumigants on EPN activity, both in production potato fields and under controlled conditions in laboratory and greenhouse microcosms.

Our project had two objectives. First, using sentinel waxworm (*Galleria mellonella* L.) hosts, EPN infectivity was measured in production potato fields in the Columbia Basin of Washington State, USA, that differed in pest management regime (certified organic versus conventional) and in the use of mustard green manure. These data suggested that mustard green manures might harm endemic EPNs. Therefore, laboratory and greenhouse-microcosm assays were designed to examine the impacts of extracts (in the laboratory trial) and soil-incorporated foliage (in the greenhouse-microcosm trial) from two *Brassica juncea* (L.) cultivars, differing in glucosinolate concentrations, on *G. mellonella* infection by several *Steinernema* and *Heterorhabditis* EPN species commercially available for use as bio-insecticides.

2. Materials and methods

2.1. EPN infectivity in production potato fields

Using waxworm (*G. mellonella*) larvae as sentinel hosts (Kaya and Stock, 1997), we surveyed 23 production potato fields in the Columbia Basin of Washington for EPN infectivity. All fields were surveyed in July (from 14 to 23 July in 2004, and from 11 to 26 July in 2005; Table 1). The fields varied both in pest management regime, certified organic versus conventional, and in mustard treatment, mustard green manure crop soil-amended in the fall preceding the potato crop or no mustard green manure used (Table 1). The soil composition of the Columbia Basin is predominately Quincy sand and Shano silt loam (Lenfesty, 1967; Gentry, 1984). All fields were irrigated using a center pivot irrigation system. Fertility of organic potato fields was enhanced using composted cow and chicken manure, whereas the conventionally managed potato fields received synthetic fertilizers equivalent to rates in Lang et al. (1999). The organic growers refrained from using insecticides but utilized foliar-applied copper fungicides (personal comm. Brad Bailey, Lenwood Farms; and Stacy Kniveton, Johnson Agriprises). The conventional potato growers utilized several synthetic pesticides including seed coat fungicides (e.g. flutolanil), copper fungicides, lateblight and white mold control (e.g. boscalid, fluazinam, and azoxystrobin), and occasionally insecticides (including some or all of the following: methamidophos, pymetrozine, and esfenvalerate) (personal communication Stacy Kniveton, Johnson Agriprises; Gilbert Hintz, Ephrata Farms; and Troy Grimes, Watts Brothers Farms). Organic fields not receiving mustard green manure were virgin ground that received no soil fumigant, whereas conventional no-mustard fields were treated with soil applications of aldicarb and metam potassium. Fields utilizing mustard green manure planted Caliente® brand mustard, which is a blend of *B. juncea* and *S. alba,* while conventional fields also incorporated soil applications of aldicarb (Table 1). Mustard green manure was planted mid-August, and then chopped and double-disked into the soil from late-October to the first week of November. Only a pre-plant fertilizer and water were applied to maintain the mustard green manure crop (personal communication Stacy Kniveton, Johnson Agriprises).

Groups of five *G. mellonella* larvae (Sunshine mealworms, Silverton, OR) were placed in mesh bags made from fiberglass window screen (Phifer®, Tuscaloosa, AL), closed and sealed with a twist tie. Ten of these bags were placed in each field in a single linear transect, with bags spaced 9.14 m apart. Sentinel hosts were buried 10–15 cm under the soil, reproducing the depth at which CPB pupate in the soil (Hare, 1990), for 48 h, after which the sentinel hosts were retrieved and returned to the laboratory. Once in the laboratory dead waxworms were placed, individually, onto modified White traps (White, 1927). These larvae were then monitored daily, for 1 week, for infection by EPNs which were identified to genus using the distinctive color of infected hosts. Insects infected by *Heterorhabditis* species turn a red-brown indicative of infection by the symbiotic *Photorhabdus* bacteria associated with EPNs in this genus (Boemare et al., 1996). Insects infected by *Steinernema* species assume a tan to gray appearance indicative of infection by the symbiotic *Xenorhabdus* bacteria associated with EPNs in that genus (Boemare et al., 1996). To verify that nematodes were capable of killing Colorado potato beetles, EPNs that we recovered from the sentinel waxworm hosts were collected from each White trap. EPN isolates, from subsamples of the same field, were then mixed together in de-ionized water to create separate suspensions for members of each EPN genus. We then added 1 ml (50 infective juveniles) of these EPN suspensions separately to different cells of 24-well tissue culture trays (Greiner Bio-One, Monroe, NC) containing 1 g of sterilized sand and one field-collected 4th instar (last stage) Colorado potato beetle larva. After 2 weeks these beetles were dissected to determine mortality by EPNs.

2.2. Effects of mustard extracts on EPNs in laboratory arenas

Our field measurements of EPN infectivity suggested that the use of mustard green manures might have negative effects on endemic EPNs (see Section 3). However, in these field measurements, a broad range of management practices and environmental factors differed among potato fields irrespective of mustard green manure treatment (Table 1), adding substantial variability to the data and rendering it impossible to entirely isolate any effects of mustard green manures. Thus, we conducted assays in the laboratory wherein we compared the effects of extracts from two *B. juncea* cultivars that differed in their glucosinolate concentrations on a diverse group of *Heterorhabditis* and *Steinernema* species. Our goals were to determine the impacts of mustards on *G. mellonella* infection by the EPN species, whether impacts differed between members of the two EPN genera, and whether impacts differed between the two mustard cultivars.

We examined two cultivars of *B. juncea*, 'Arid' and 'Pacific Gold'. 'Arid' has lower glucosinolate levels, ca. 10 μmol g^{-1} (Malhi et al., 2007), than 'Pacific Gold' which has higher glucosinolate levels, ca. 300 μmol g^{-1} (Brown et al., 2004). We planted 10 plants of each of the two mustard cultivars in the greenhouse (16:8 h, light:dark cycle; 27 °C) and 30 days later, just prior to flowering, chopped the plants into ca. 1.25-cm pieces using scissors. This reproduced the

Table 1
Characteristics of the potato fields where entomopathogenic nematode infectivity was measured.

Regime	Soil treatment	Hectares	County	Grower	Year
Conventional	Metam sodium	48.56	Grant	Ephrata	2004
Conventional	Aldicarb and metam potassium	48.56	Adams	Johnson	2004
Conventional	Metam sodium	48.56	Grant	Ephrata	2005
Conventional	Aldicarb and metam potassium	48.56	Adams	Johnson	2005
Conventional	Aldicarb and metam potassium	24.28	Adams	Johnson	2005
Conventional	Aldicarb and metam potassium	48.56	Adams	Johnson	2005
Conventional	Aldicarb and metam potassium	48.56	Adams	Johnson	2005
Conventional	Metam sodium, 1, 3-dichloropropene	48.56	Benton	Watts Bros.	2005
Conventional	Mustard green manure and aldicarb	24.28	Adams	Johnson	2004
Conventional	Mustard green manure and aldicarb	48.56	Adams	Johnson	2004
Conventional	Mustard green manure	18.21	Benton	Paterson	2004
Conventional	Mustard green manure	18.21	Benton	Paterson	2004
Conventional	Mustard green manure and metam sodium	48.56	Benton	Watts Bros.	2004
Conventional	Mustard green manure and aldicarb	24.28	Adams	Johnson	2005
Conventional	Mustard green manure and aldicarb	24.28	Adams	Johnson	2005
Conventional	Mustard green manure and aldicarb	48.56	Adams	Johnson	2005
Conventional	Mustard green manure	18.21	Benton	Paterson	2005
Organic	None	24.28	Franklin	Lenwood	2004
Organic	None	24.28	Adams	Johnson	2005
Organic	None	24.28	Franklin	Lenwood	2005
Organic	None	24.28	Franklin	Lenwood	2005
Organic	Mustard green manure	24.28	Adams	Johnson	2004
Organic	Mustard green manure	24.28	Adams	Johnson	2005

typical practice in production potato fields, wherein mustard plants are chopped and tilled into the soil just before flowering to maximize bio-fumigation (McGuire, 2003b). We then prepared extracts from each mustard cultivar by blending 10 g of fresh plant material (stems and leaves) with 100 ml of de-ionized water. The resulting slurry was sieved to obtain an aliquot of extract suspension (Matthiessen and Shackleton, 2005). Suspensions prepared in this way captured biologically active plant compounds because maceration of plant tissues releases glucosinolate and myrosinase compounds, which are water-soluble (Brown and Morra, 1997). Biofumigant effects may not be derived from the glucosinolates directly but from the enzymatic degradation of glucosinolates by myrosinase in the presence of water (Brown and Morra, 1995). Mustard suspensions were created separately, and used immediately thereafter, for each of the two experiments described below.

The first experiment included three *Steinernema* species: *Steinernema carpocapsae*, *Steinernema glaseri*, and *Steinernema feltiae*. The second experiment included the above species, an additional *Steinernema* species (*Steinernema riobrave*), and three *Heterorhabditis* species (*Heterorhabditis bacteriophora*, *Heterorhabditis marelatus*, and *Heterorhabditis megidis*). These EPN species were selected because they are available commercially or have been examined for use as biopesticides (Grewal, 2002), and because all but *H. megidis* have been investigated for use in Colorado potato beetle biological control (MacVean et al., 1982; Toba et al., 1983; Wright et al., 1987; Cantelo and Nickle, 1992; Berry et al., 1997; Stewart et al., 1998; Armer et al., 2004). *S. glaseri*, *H. bacteriophora*, and *H. megidis* were purchased from a commercial supplier (Integrated Fertility Management, Wenatchee, WA), whereas *S. feltiae*, *S. riobrave*, *S. carpocapsae* and *H. marelatus* came from laboratory cultures using *G. mellonella* larvae as hosts (Riga et al., 2006).

Each EPN species was subjected to each of three mustard extract treatments: CONTROL, no mustard extract; ARID (extract from *B. juncea* cv. 'Arid'), and GOLD (extract from *B. juncea* cv. 'Pacific Gold'). The first experiment included five replicates of each EPN species/mustard treatment combination (total $N = 45$), whereas the second experiment included 10 replicates of each EPN species/mustard treatment combination (total $N = 210$). Our experimental arenas were 9-cm-diameter Petri dishes lined with filter paper. GOLD and ARID treatments received 1 ml of aqueous suspension of *B. juncea* extract, freshly blended and sieved as described above from the appropriate *B. juncea* cultivar; CONTROL replicates received 1 ml of de-ionized water. Thereafter, we immediately applied EPNs at a rate of 250 infective juveniles per dish. Petri dishes were then left undisturbed for 24 h (at 20–24 °C), allowing time for mustard extracts to impact the nematodes, before five *G. mellonella* larvae were placed into each arena. These larvae were left in the dishes for 1 week, after which each larva was scored for infection by EPNs as previously described.

2.3. Effects of green manures on EPNs in greenhouse soil-plant microcosms

Our Petri-dish assays had two limitations: (1) they did not include soil, which can dramatically alter the impact of mustard extracts on nematodes (e.g. Matthiessen and Shackleton, 2005), and (2) they examined the effects of mustard extracts rather than plant foliage breaking down in soil. Therefore, we repeated our mustard-exposure trials in larger pot arenas in the greenhouse wherein we grew, chopped, and incorporated green manures into field-collected soil. Also, in our earlier laboratory trial we could not distinguish between the effects of mustard green manures per se, versus a more general effect of plant extracts on EPNs. Thus, we also added a treatment where perennial ryegrass (*Lolium perenne* L.) was grown and incorporated into the soil as an additional control.

In these experiments we included two *Steinernema* species (*S. carpocapsae* and *S. feltiae*) and three *Heterorhabditis* species (*H. bacteriophora*, *H. marelatus*, and *H. megidis*). Each EPN species was subjected to each of the following four treatments: CONTROL, soil and water only; BIOMASS CONTROL, soil incorporation of perennial ryegrass foliage; ARID, soil incorporation of *B. juncea* cv. 'Arid' foliage; and GOLD, soil incorporation of *B. juncea* cv. 'Pacific Gold' foliage. This experiment was conducted twice, representing TRIAL 1 and TRIAL 2, respectively. Each trial included five replicates per nematode species/soil treatment combination for a total of 100 replicate microcosms per trial, and 200 replicate microcosms across the two trials.

Our experimental units were 800-ml-capacity pots filled with one part potting soil to three parts un-sterilized field-collected soil (Shano silt loam, pH 7.7) with the field soil gathered from Washington State University's Research and Education Center in Othello,

Washington. GOLD and ARID replicates received 12 mg of mustard seed/pot, while BIOMASS CONTROL replicates received 24 mg of perennial ryegrass seed, with both seeding rates approximating typical practices when these green manures are grown preceding Washington potato crops (McGuire, 2003b). The green manure plants were grown in a greenhouse on the campus of Washington State University under a 16:8 h, light:dark cycle at 27 °C. Pots were bottom-watered once a week by adding water to the individual trays containing each pot. Four weeks later, when mustard plants were ca. 52 cm tall (18 g of aboveground plant biomass/pot), prior to flowering, and ryegrass plants were ca. 14 cm tall (8 g of aboveground plant biomass/pot), green manure plants were cut at the base, chopped, and thoroughly incorporated into the soil and roots. Soil was also mixed in CONTROL replicates, but no plant biomass was incorporated. Thereafter, we immediately applied EPNs at a rate of 6.17×10^5 infective juveniles/m^2. Next, 1, 7 and 14 days after EPN release, groups of five sentinel *G. mellonella* larvae were placed into each microcosm to track the course of EPN infectivity through time. *G. mellonella* were added to the soil surface of each pot and confined using an inverted cup (Solo®, 207-ml plastic cup, Highland Park, IL) with the bottom cut out and the cup rim fixed to the soil surface. Sentinel larvae were left in the pots for 48 h, collected, and scored for infection by EPNs as previously described.

2.4. Analyses

The field data were analyzed within a 2×2-factorial design in ANOVA, with two levels of pest management (Organic, Conventional) and two levels of mustard treatment (Applied, Not applied).

Infection data from the first laboratory experiment were analyzed within a two-way ANOVA structure with three levels of mustard treatment (CONTROL, ARID, and GOLD) fully crossed with the three nematode species (*S. carpocapsae*, *S. glaseri*, and *S. feltiae*). For the second laboratory experiment, mustard and species effects were nested within the two genera (*Heterorhabditis* and *Steinernema*). The greenhouse-microcosm experiments (TRIAL 1 and TRIAL 2) were analyzed separately within repeated measures two-way ANOVA with four levels of soil treatment (CONTROL, BIOMASS CONTROL, ARID, and GOLD) crossed with the five EPN species (all species but *S. glaseri* and *S. riobrave* were included in this experiment); soil-treatment and EPN species effects were nested within the two genera (*Heterorhabditis* and *Steinernema*). For both laboratory and greenhouse experiments, within the full experimental design green manure treatment main effects (or interactions between the green manure treatments and EPN species or genus) could be due to differing EPN infectivity in the presence versus absence of green manures, differing EPN infectivity in the presence of the two mustard cultivars, or both. Thus, we followed our initial analysis with two planned, additional tests. First, within the complete multi-factorial design we pooled data from the two mustard addition treatments, to yield two levels of mustard manipulation (MUSTARD+, MUSTARD−); for the greenhouse-microcosm experiment MUSTARD− consisted of the pooled CONTROL and BIOMASS CONTROL treatments. Second, we compared the impacts of the two mustard cultivars and their interactions with EPN taxa by dropping the CONTROL treatment (and also the BIOMASS CONTROL treatment in the greenhouse-microcosm experiment) from the model, again yielding two levels of mustard manipulation (ARID and GOLD). An additional contrast for the greenhouse-microcosm experiment tested the addition of plant biomass on EPN infection by dropping ARID and GOLD treatments from the model and comparing the CONTROL to BIOMASS CONTROL treatments.

All proportion infection data were arcsine square-root transformed prior to analysis. Analyses of data from the field study, the first laboratory experiment, and the greenhouse experiment were analyzed using SYSTAT (version 11.0; SPSS, Chicago, IL) software. Data from laboratory experiment 2 were analyzed using SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. EPN infectivity in production potato fields

There was a trend for overall rates of EPN infection on *G. mellonella* to be lower in production potato fields receiving mustard green manure (mustard main effect: $F = 2.88$; $df = 1,19$; $P = 0.106$; Fig. 1A). However, effects of pest management regime ($F = 0.01$; $df = 1, 19$; $P = 0.950$), and the interaction between mustard green manure and pest management regime ($F = 1.74$; $df = 1,19$; $P = 0.203$), were clearly not significant. Sentinel *G. mellonella* larvae were infected by members of the EPN genera *Heterorhabditis* and *Steinernema*. Across all field types the majority of *G. mellonella* infections were by *Heterorhabditis* species, representing 56% of all *G. mellonella* infected by EPNs. Between the EPN genera, activity of *Heterorhabditis* species exhibited the greatest magnitude of difference among field types (Fig. 1B). However, mustard treatment and pest management regime main and interactive effects were not statistically significant for *Heterorhabditis* (mustard main effect: $F = 1.66$; $df = 1,19$; $P = 0.214$; management regime main effect: $F = 0.20$; $df = 1, 19$; $P = 0.660$; interaction: $F = 1.72$; $df = 1,19$; $P = 0.205$) or *Steinernema* (mustard main effect: $F = 0.38$; $df = 1,19$; $P = 0.548$; management regime main effect: $F = 1.63$; $df = 1,19$; $P = 0.217$; interaction: $F = 0.13$; $df = 1,19$; $P = 0.720$) species when these EPN genera were analyzed separately (Fig. 1B and C).

In total, we collected 35 *Steinernema* and 95 *Heterorhabditis* isolates from the field-placed sentinel waxworms. Of these, 71% of *Steinernema* and 74% of *Heterorhabditis* isolates proved capable of killing Colorado potato beetle larvae. While 75% beetle mortality was recorded overall, 42% of all beetle death was attributed to EPNs. In particular, two *Steinernema* isolates from each of one

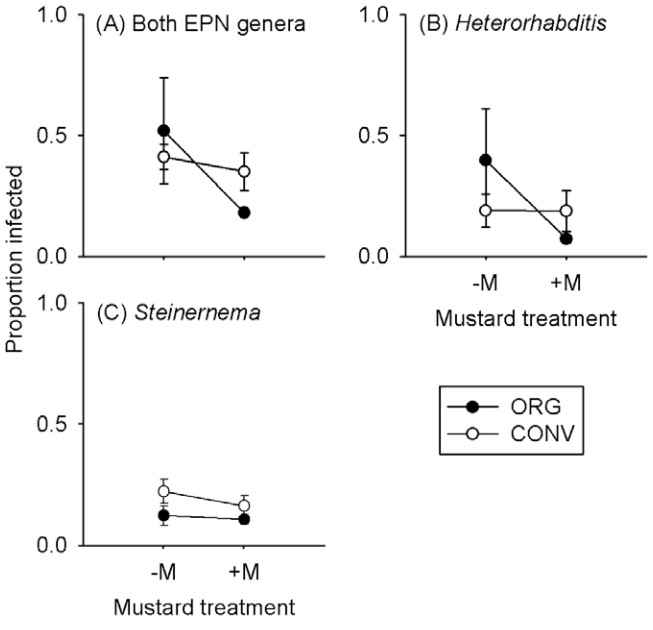

Fig. 1. Proportion of sentinel *Galleria mellonella* hosts infected by (A) both entomopathogenic nematode (EPN) genera, (B) *Heterorhabditis* spp. nematodes, and (C) *Steinernema* spp. nematodes. Mustard treatments: mustard green manure not used (−M), mustard green manure used (+M). Pest management regime: conventional pest management (○), certified organic (●). Means are +1SE.

organic (Lenwood) and one conventional (Johnson) field recorded greater than 80% beetle mortality.

3.2. Effects of mustard extracts on EPNs in laboratory arenas

In the first laboratory experiment we examined the impact of two *B. juncea* cultivars (the low glucosinolate cultivar 'Arid' and the high glucosinolate cultivar 'Pacific Gold'), compared to a water control, on infection of *G. mellonella* larvae by three *Steinernema* species (*S. carpocapsae*, *S. feltiae* and *S. glaseri*). Across the complete experimental design, we found a significant mustard treatment x EPN species interaction ($F = 4.87$; $df = 4,81$; $P = 0.001$). To further investigate the nature of this interaction, we conducted two additional tests. When combining treatments receiving mustard extract from the two cultivars (pooled ARID + GOLD), we also found a significant mustard treatment × EPN species interaction ($F = 6.41$; $df = 2,84$; $P = 0.003$; Fig. 2A). This interaction appeared to be driven by the high infectivity of *S. feltiae* regardless of mustard extract addition (Fig. 2A); EPN infectivity generally declined in the presence of mustard extract (mustard main effect: $F = 8.70$; $df = 1,84$; $P = 0.004$; Fig. 2A). When we eliminated the CONTROL treatment from the analysis to highlight comparison between the two *B. juncea* cultivars, the mustard treatment × EPN species interaction remained statistically significant ($F = 3.22$; $df = 2,54$; $P = 0.048$; Fig. 2A), an effect again apparently driven by the consistently strong performance of *S. feltiae* across extracts of the two *B. juncea* cultivars (Fig. 2A). EPN species generally exhibited poorer *G. mellonella* infectivity in extract from the high-glucosinolate cultivar 'Pacific Gold' than in extract from the low-glucosinolate cultivar 'Arid' (mustard main effect: $F = 4.43$; $df = 1,54$; $P = 0.040$), and species significantly differed in overall *G. mellonella* infectivity (EPN species main effect: $F = 7.71$; $df = 2,54$; $P = 0.001$; Fig. 2A).

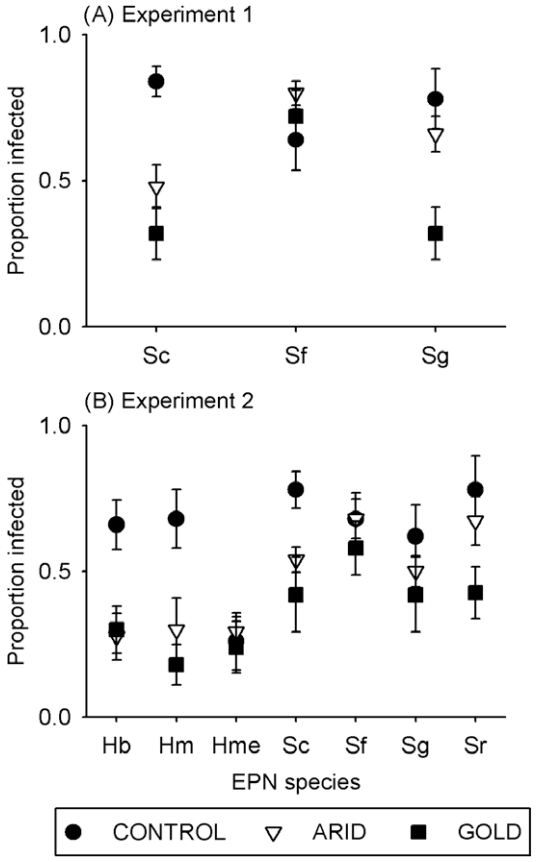

Fig. 2. For (A) laboratory experiment 1 and (B) laboratory experiment 2, proportion of *Galleria mellonella* hosts infected by entomopathogenic nematodes (EPNs). EPN species treatments: *H. bacteriophora* (Hb), *H. marelatus* (Hm), *H. megidis* (Hme), *S. carpocapsae* (Sc), *S. feltiae* (Sf), *S. glaseri* (Sg), and *S. riobrave* (Sr). Mustard treatments: water (CONTROL), extract from *B. juncea* cv. 'Arid' (ARID), and extract from *B. juncea* cv. 'Pacific Gold' (GOLD). Means are +1SE.

Upon measuring the impact of *B. juncea* extracts on four *Steinernema* (*S. carpocapsae*, *S. feltiae*, *S. glaseri* and *S. riobrave*) and three *Heterorhabditis* (*H. bacteriophora*, *H. marelatus* and *H. megidis*) species, within the complete experimental design, we found that mustard treatment ($F = 15.16$; $df = 2,189$; $P = 0.001$) and EPN genus ($F = 30.37$; $df = 1,\ 189$; $P = 0.001$) exerted statistically significant impacts on EPN infectivity (Fig. 2B); all other main and interactive effects were not statistically significant (EPN species main effect: $F = 1.29$; $df = 5,189$; $P = 0.271$; mustard treatment × genus interaction: $F = 0.89$; $df = 2,189$; $P = 0.413$; mustard treatment × EPN species interaction: $F = 1.31$; $df = 10,189$; $P = 0.230$). Pooling the two mustard extract treatments and comparing them to the water control revealed a significant mustard treatment × EPN species interaction ($F = 2.35$; $df = 5,196$; $P = 0.043$), indicating that species differed in their response to mustard extracts. Again, this interaction appeared to be influenced by the relatively robust performance of *S. feltiae* in both the presence and absence of mustard extract, and by the consistently poor performance of *H. megidis* across treatments (Fig. 2B). Results for these two species run counter to the general trend of lower EPN infection rates with the addition of mustard extracts across all EPN species (mustard main effect: $F = 23.96$; $df = 1,196$; $P < 0.001$; Fig. 2B). *Steinernema* species exhibited generally higher infection rates than did *Heterorhabditis* species (genus main effect: $F = 30.21$; $df = 1,196$; $P < 0.001$), an effect not influenced by mustard addition (genus × mustard interaction: $F = 1.20$; $df = 1,196$; $P = 0.276$). When we compared ARID to GOLD treatments minus the CONTROL, the analysis revealed that EPN species generally performed more poorly when exposed to extracts from the high glucosinolate mustard cultivar 'Pacific Gold' than the low glucosinolate cultivar 'Arid' (mustard main effect: $F = 6.54$; $df = 1,126$; $P = 0.012$), and that *Steinernema* species again outperformed *Heterorhabditis* species (genus main effect: $F = 27.64$; $df = 1,126$; $P < 0.001$; Fig. 2B). However, interactions between mustard cultivar and EPN species and genus were not statistically significant (mustard treatment × EPN species interaction: $F = 0.26$; $df = 5,126$; $P = 0.933$; mustard treatment x genus interaction: $F = 0.60$; $df = 1,126$; $P = 0.440$), suggesting that on average EPN species were similarly harmed by the high glucosinolate *B. juncea* cultivar.

3.3. Effects of green manures on EPNs in greenhouse soil-plant microcosms

In TRIAL 1, across the complete experimental design, a significant time × soil treatment × EPN genus interaction ($F = 2.71$; $df = 6,160$; $P = 0.015$) was observed, indicating that overall treatment effects grew in intensity at different rates for the two EPN genera. To further investigate this interaction, we pooled ARID and GOLD treatments and compared these to the pooled controls (CONTROL + BIOMASS CONTROL). Here, we found significant time × mustard × EPN genus ($F = 3.97$; $df = 2,180$; $P = 0.021$), and time × mustard × EPN species ($F = 2.13$; $df = 6,180$; $P = 0.052$), interactions (Fig. 3A–E). These interactions appear to reflect a relatively dramatic increase in the negative effects of mustards on *Heterorhabditis* species through time, compared to a more modest reduction in activity through time for the two *Steinernema* species taken together (Fig. 3) and for *S. feltiae* in particular (Fig. 3E). The highly significant mustard main effect ($F = 104.2$; $df = 1,90$; $P < 0.001$) indicated that, overall, mustard addition was harmful to EPN infectivity. Comparison between CONTROL and BIOMASS CONTROL treatments showed no significant differences ($F = 2.59$;

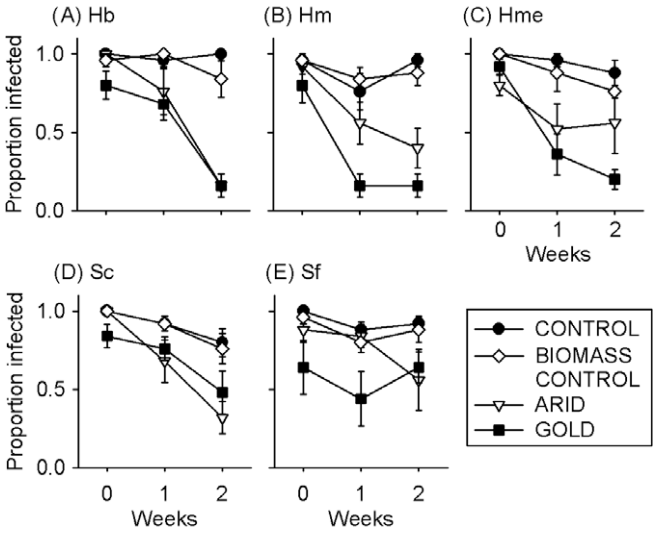

Fig. 3. Proportion of *Galleria mellonella* hosts infected by entomopathogenic nematodes (EPNs) 24 h, 1 week, and 2 weeks after green manure and EPN application in trial 1. EPN species treatments: (A) *H. bacteriophora* (Hb), (B) *H. marelatus* (Hm), (C) *H. megidis* (Hme), (D) *S. carpocapsae* (Sc), and (E) *S. feltiae* (Sf). Mustard treatments: water (CONTROL), green manure from perennial ryegrass (BIOMASS CONTROL), green manure from *B. juncea* cv. 'Arid' (ARID), and green manure from *B. juncea* cv. 'Pacific Gold' (GOLD). Means are +1SE.

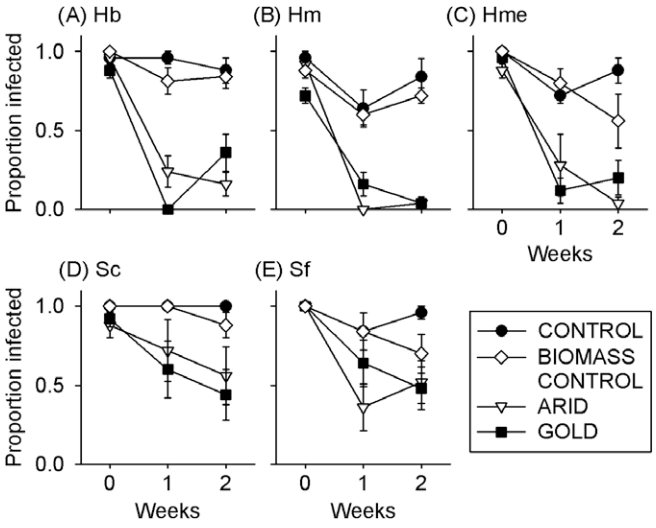

Fig. 4. Proportion of *Galleria mellonella* hosts infected by entomopathogenic nematodes (EPNs) 24 h, 1 week, and 2 weeks after green manure and EPN application in trial 2. EPN species treatments: (A) *H. bacteriophora* (Hb), (B) *H. marelatus* (Hm), (C) *H. megidis* (Hme), (D) *S. carpocapsae* (Sc), and (E) *S. feltiae* (Sf). Mustard treatments: water (CONTROL), green manure from perennial ryegrass (BIOMASS CONTROL), green manure from *B. juncea* cv. 'Arid' (ARID), and green manure from *B. juncea* cv. 'Pacific Gold' (GOLD). Means are +1SE.

$df = 1,40$; $P = 0.115$), suggesting that EPNs were not harmed by the addition of ryegrass foliage. To compare effects of the two *B. juncea* cultivars, we next dropped the controls (both CONTROL and BIOMASS CONTROL) from the analysis while retaining the factors ARID and GOLD. Here, we detected significant time × EPN species ($F = 3.27$; $df = 6, 80$; $P = 0.006$) and time × EPN genus ($F = 3.49$; $df = 2,80$; $P = 0.035$) interactions (Fig. 3A–E), again apparently driven by the increasingly deleterious effects of 'Pacific Gold' on *Heterorhabditis* species through time, and the relative insensitivity of *Steinernema* generally and *S. feltiae* in particular to these effects (Fig. 3E). Additionally, the time × EPN species interaction may have been influenced by the relatively modest difference between the two mustard cultivars in harmful effects to *H. bacteriophora* (Fig. 3A) and *S. carpocapsae* (Fig. 3D). The significant mustard cultivar main effect ($F = 6.72$; $df = 1,40$; $P = 0.013$), and non-significant interactions between mustard cultivar and other model factors, suggest that, on the whole, the high glucosinolate mustard cultivar 'Pacific Gold' was more damaging to these EPN species than was the low glucosinolate cultivar 'Arid'.

The results from TRIAL 2 generally mirrored those of TRIAL 1 (Fig. 4). We again found a significant time × soil treatment × EPN genus interaction ($F = 3.18$; $df = 6,160$; $P = 0.006$) in the overall analysis, while TRIAL 2 also revealed a significant time × soil treatment × EPN species interaction ($F = 1.97$; $df = 18,160$; $P = 0.014$). When comparing the pooled mustard addition (ARID + GOLD) to the pooled controls (CONTROL + BIOMASS CONTROL), as in TRIAL 1 we found a significant time × mustard × EPN genus interaction ($F = 3.65$; $df = 2,180$; $P = 0.028$; Fig. 4) and mustard main effect ($F = 162.9$; $df = 1,90$; $P < 0.001$). Unlike the first trial, however, comparison between CONTROL and BIOMASS CONTROL showed a significant time × soil treatment interaction ($F = 4.63$; $df = 2,80$; $P = 0.013$), driven by a slight decrease in EPN infection with the addition of ryegrass foliage in the second week of the trial (Fig. 4). In comparing the effects of ARID and GOLD in TRIAL 2, the non-significant mustard main effect ($F = 2.59$; $df = 1,40$; $P = 0.115$) and significant time × mustard × EPN species ($F = 2.64$; $df = 6,80$; $P = 0.022$) and time × mustard × EPN genus interactions ($F = 3.99$; $df = 2,80$; $P = 0.022$), apparently resulted from the immediate harmful effects of both mustard cultivars on *Heterorhabditis* species in the first week (Fig. 4A–C) and relative resistance to both mustard cultivars by *S. carpocapsae* and *S. feltiae* through time (Fig. 4D–E).

4. Discussion

In production potato fields, EPN infection of *G. mellonella* on average was ca. 20% less common in fields receiving mustard green manure (Fig. 1A), an effect not statistically significant. EPNs in the genus *Heterorhabditis* were the most commonly collected in our field sampling, representing 56% of all EPN infections of *G. mellonella*. *Heterorhabditis* species infections on *G. mellonella* were on average 35% less common in mustard treated fields, with the most dramatic reduction apparent for organic fields (Fig. 1B); however, the data were highly variable and this reduction was not statistically significant. This variability in the field data likely stems from the many differences in management practices, such as differences in insecticide and fungicide products used and the intensity of this usage by our cooperating growers (Table 1), in addition to inherent differences among fields in rotational history, physical soil properties, pH and other environmental characteristics. We made no attempt to identify field-collected EPN isolates to species, but found that most isolates of both *Steinernema* and *Heterorhabditis* species were capable of infecting Colorado potato beetles. Thus, harmful effects of mustard green manures on EPNs have the potential to harm biological control of potato beetles and perhaps other soil-dwelling pest insects.

In our laboratory and greenhouse bioassays, exposure to *B. juncea* generally reduced the likelihood that EPNs would infect *G. mellonella* larvae (Figs. 2 and 3), supporting the trend in the field survey (Fig. 1A). Several lines of evidence suggest that mustard foliage reduced EPN infectivity. First, exposure to extract from the high-glucosinolate *B. juncea* cv. 'Pacific Gold' generally had a greater negative effect on EPN performance than did extract from the low-glucosinolate content cultivar 'Arid' (Figs. 2A, B and 3B, C). This is consistent with glucosinolates, or their toxic breakdown products, acting as antagonists to nematodes (Zasada and Ferris,

2004). Second, EPN infectivity was not affected (greenhouse TRIAL 1), or only weakly harmed (greenhouse TRIAL 2), by the soil-incorporation of ryegrass, indicating that EPN infectivity was not strongly impacted by the addition of non-mustard plant biomass. Thus, mustard green manures may be particularly harmful to EPNs.

In the laboratory and greenhouse trials *Heterorhabditis* species generally performed more poorly than did *Steinernema* species and appeared also to be more susceptible to the harmful effects of mustard green manures. This trend corresponds with the results of the field survey, wherein *Heterorhabditis*, but not *Steinernema*, species showed a trend towards lower densities in the presence of mustard green manure treatment (Fig. 1B–C). Furthermore, among *Steinernema* species, *S. feltiae* exerted relatively high infection rates in the presence of *B. juncea* extracts/foliage (Figs. 2A and B, 3E, and 4E). *Steinernema* species generally, and *S. feltiae* in particular, may warrant particular attention for use as a bio-pesticide in potato or other cropping fields where mustard bio-fumigants are also used. Currently, the mode of action of mustard bio-fumigants on nematodes is unknown (Sipes and Schmitt, 1998), and thus the mechanism underlying the apparent low susceptibility of *Steinernema* species (and *S. feltiae* in particular) to mustard green manures is unclear and likely warrants greater study. However, it is possible that these EPNs possess biological traits that render them inherently more resistant to the harmful effects of mustard green manures. Similarly, different species of plant–parasitic nematodes vary in their susceptibility to mustard bio-fumigants (Zasada and Ferris, 2004).

Generally speaking, we observed remarkable concordance between results from the Petri dish trials, and those conducted in greenhouse microcosms incorporating *in situ* growth, chopping, and soil incorporation of mustard green manures. For example, in both of the laboratory experiments, and again at the later sample points in the greenhouse experiment, *H. bacteriophora* exhibited clearly reduced activity in the presence of mustards, but no difference in the effect of the low versus high glucosinolate mustard cultivars (Figs. 2, 3A and 4A). Similarly, *S. feltiae* exhibited no apparent harm from mustard extracts in either laboratory trial (Fig. 2), and relatively weakly reduced activity in the presence of mustards in the greenhouse-microcosm trials (Figs. 3E and 4E). Our data suggest that the laboratory-exposure protocol may be a relatively accurate means to screen EPN species and strains for their susceptibility to the effects of different green manure crops. However, whereas harmful effects of mustard extracts were immediately observable in the Petri dish arenas, negative effects of soil-incorporated mustard foliage developed more gradually in the more-realistic greenhouse microcosms. The time course of mustard effects on EPNs may be important in the field, and likely will not be well-replicated in simple trials where EPNs are exposed to pure mustard extracts suspended in water.

Governments have restricted the use of synthetic soil fumigants such as methyl bromide, metam sodium, and 1,3-dichloropropene, due to these chemicals' substantial environmental and human-health risks. These concerns have led to an ongoing search for effective alternatives. In addition to *Brassica* and *Sinapis* mustard species, sudan grass (Mojtahedi et al., 1993), oat and rye (Faulkner and McElroy, 1964), and forage millet and marigolds (Ball-Coelho et al., 2003; Riga et al., 2005) have been examined as rotational or green manure crops as a tactic to reduce buildup of plant-parasitic nematode populations. Additional advantages of bio-fumigants include little or no risk to the environment or to humans, reduced soil erosion, improved soil fertility, and sometimes additional income for growers (Matthiessen and Kirkegaard, 2006). Mustards have been particularly attractive bio-fumigant candidates because of the broad activity of their toxic breakdown products against a range of soil pests (Brown and Morra, 1995; Kirkegaard et al., 1996; Zasada and Ferris, 2004). Furthermore, biologically-active compounds are retained in waste-products following conversion of mustard seed to biofuels, forming an inexpensive and likely growing source of these soil amendments (Cohen and Mazzola, 2004).

However, this same broad toxicity may carry tradeoffs. Beneficial bacteria, fungi, and EPNs form an important component of good soil health that could also be harmed by the use of mustard bio-fumigants. Our work suggests a particular conflict between mustard bio-fumigation for the control of plant-parasitic nematodes, and the conservation and/or augmentation of EPNs for biological control. Simply increasing the period of time between bio-fumigation and the application of EPN bio-pesticides appears unlikely to entirely mitigate these harmful effects, as our field sampling suggested reduced EPN infectivity approximately 8 months after the incorporation of mustard green manures. Similarly, in the greenhouse-microcosm study harmful effects grew more distinct through the several weeks of the trials. A more promising approach may be to instead focus on augmentation and conservation of EPN species that, like *S. feltiae*, are relatively tolerant of any harmful effects of mustard green manures.

EPNs have a long record of showing effective pest control in laboratory arenas, but often limited efficacy in the field (Klein, 1990; Smith, 1999). Rotational and green manure crops (this study), soil fertility practices (Duncan et al., 2007), biotic resistance by other soil organisms (Kaya and Koppenhöfer, 1996), and tillage intensity (Millar and Barbercheck, 2001) all have impacts on entomopathogen communities in the soil. Thus, maximizing the effectiveness of beneficial entomopathogens through their conservation and augmentation likely requires a systems-based approach, balancing tradeoffs among the full range of soil-management tactics.

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