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Control of *Globodera* spp. Using *Brassica juncea* Seed Meal and Seed Meal Extract

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Abstract: The eradication program for the potato cyst nematode (PCN), *Globodera pallida*, in the Northwest of the United States revolves around the use of soil fumigation. Alternative, integrated strategies are needed to continue to battle this invasive nematode. Laboratory, greenhouse, and field experiments were conducted with *G. pallida* and another cyst nematode found in the United States, *Globodera ellingtonae*, to evaluate the efficacy of a new formulated *Brassica juncea* seed meal extract, as well as a traditional *B. juncea* seed meal, as alternate eradication strategies. This is the first report on the efficacy of *B. juncea* seed meal extract against plant-parasitic nematodes. Rates of *B. juncea* seed meal greater than 2.2 t/ha and 4.5 t/ha for *G. pallida* and *G. ellingtonae*, respectively, were required for egg hatch suppression, as determined by a potato root diffusate (PRD) bioassay. Reproduction of *G. pallida* on potato after exposure to *B. juncea* seed meal at a rate of 2.2 t/ha was also significantly reduced. In the field, 8.9 t/ha *B. juncea* seed meal almost eliminated egg hatch of *G. ellingtonae*. Rates needed for *Globodera* spp. suppression were greatly reduced when using the *B. juncea* seed meal extract. When compared side-by-side, half as much *B. juncea* seed meal extract, 1.1 t/ha, was required to suppress *G. ellingtonae* egg hatch to the same extent as *B. juncea* seed meal. Exposure of *G. pallida* to *B. juncea* seed meal extract at 4.5 t/ha reduced egg hatch by 90% compared with a nonamended control. The ability to reduce the amount of material being applied to soil by using an extract has the potential for integration into a *G. pallida* eradication program.

Key words: brassica, *Globodera* spp., glucosinolate, isothiocyanate, potato cyst nematode, yellow mustard.

The potato cyst nematodes (PCN), *Globodera rostochiensis* and *G. pallida*, are internationally recognized quarantine pests that are among the most damaging pests to potato, causing up to 80% yield loss (Brodie, 1984; Singh et al., 2013). Both species of PCN are found in the United States. The golden nematode, *G. rostochiensis*, is found only in New York (Chitwood et al., 1942). *Globodera pallida* is found in only two counties in Idaho (Hafez et al., 2007) and is present on less than 1% of the land in potato production in Idaho. Both are regulated by USDA-APHIS and the relevant state agency, either the Idaho State Department of Agriculture (ISDA) or the New York State Department of Agriculture. In addition to *G. pallida* and *G. rostochiensis* in the United States, another *Globodera* species, *G. ellingtonae* is found in Oregon and Idaho (Skantar et al., 2011). Because pathogenicity has not been established on potato (Zasada and Ingham, personal communication), *G. ellingtonae* is not considered a quarantine pest and therefore is not regulated.

For the United States, *G. pallida* is a quarantine pest for which there is zero tolerance. The presence of *G. pallida* in Idaho was viewed with extreme alarm by other states and countries that import Idaho potatoes. Before implementation of the current quarantine measures, import of Idaho fresh potato products and nursery stock was banned by some of Idaho's most important trading partners, including Canada, Mexico, and Japan. Consequently, eradication of *G. pallida* is a top priority for the Idaho potato industry including the Idaho Potato Commission, ISDA, and USDA-APHIS. A critical component of this work has been undertaken by USDA-APHIS through fumigation of infested fields with methyl bromide (MeBr). However, MeBr use was discontinued in 2014, and in 2016, the registrant pulled their supplemental Idaho label. USDA-APHIS currently relies on fumigation with 1,3-dichloropropene, which is not as effective, costly, and difficult to obtain.

Strategies to control plant-parasitic nematodes without the use of fumigants such as MeBr are urgently needed (Zasada et al., 2010). The narrow host range of *Globodera* spp. (Whitehead and Turner, 1998) suggests that a crop rotation of 4 years, as is used in Europe, could be effective for their control. However, *Globodera* spp. hatch only in response to specific chemicals, called hatching factors, released from potato roots or related plant species (Arntzen et al., 1993; Byrne et al., 2001; Schenk, 1999). Once hatched, second stage juvenile (J2) are attracted to a host, invade the root, form feeding sites, and then, depending on density and nutritional status of the syncytium, develop into either males or females, mate, and reproduce.

For *G. pallida*, the requirement for hatching factors is nearly absolute. In a given season, only a portion of the encysted eggs are stimulated to hatch and very few J2 hatch in the absence of a host. Consequently, population decline rates to nondetectable levels in the absence of a host can take 15 years or more for *G. pallida* (Turner, 1996; Timmermans et al., 2006) and only slightly less for *G. rostochiensis* (LaMondia and Brodie, 1986), making rotation with a nonhost impractical as an eradication strategy for infested fields in Idaho. In Europe, where both *G. pallida* and *G. rostochiensis* are prevalent in potato-growing areas, rotations of at least 4 years, the use of resistant cultivars, and testing of soil where tubers and other plants for export are grown are the primary control measures for these two important pests of potato (Hockland, 2002). Although some level of resistance to *G. pallida* is present in potato varieties grown in Europe, there is no resistance in Idaho's signature

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russet varieties, and development of such resistance is anticipated to be at least several years.

A management strategy that may be incorporated into an integrated *G. pallida* eradication program in Idaho is the use of brassicaceous seed meal. Agricultural use of biofumigation with brassicaceous amendments has been evaluated for suppression of a diversity of soilborne pests (Brown and Morra, 1997; Smolinska et al., 1997; Dandurand et al., 2000; Zasada et al., 2009; Mazzola and Brown, 2010; Lord et al., 2011; Meyer et al., 2011; Mazzola et al., 2015). Biocidal activity of brassicaceous materials, including green manures or seed meals as biofumigants, is attributed to the production of volatile toxic isothiocyanates from precursor glucosinolates as catalyzed by the enzyme myrosinase (thioglucoside glucohydrolase, EC 3.2.1.147) (Brown and Morra, 1997; Vaughn and Boydston, 1997; Buskov et al., 2002; Zasada and Ferris, 2003; Lazzeri et al., 2004). Impact of brassicaceous materials on nematodes have been extensively reviewed (Avato et al., 2013; Fourie et al., 2016), with specific reports for *Meloidogyne incognita* (Lazzeri et al., 2009; Zasada et al., 2009; Meyer et al., 2011), *Meloidogyne chitwoodi* (Mojtahedi et al., 1991), *Meloidogyne javanica* (Salem and Mahdy, 2015), *Pratylenchus penetrans* (Yu et al., 2007; Zasada et al., 2009), *G. allida*, and *G. rostochiensis* (Aires et al., 2009; Lord et al., 2011; Ngala et al., 2015; Watts et al., 2014). However, not all brassicaceous sources provide similar nematode suppression. Mustard seed meals derived from seeds contain a number of minor glucosinolates, but typically produce only one predominant type (Fahey et al., 2001). *Brassica juncea* lines containing high concentrations of 2-propenyl glucosinolate (sinigrin) that produce 2-propenyl isothiocyanate, were the most promising of the Brassicaceae species evaluated for management of *G. pallida* by biofumigation (Aires et al., 2009; Lord et al., 2011; Brolsma et al., 2014).

Inconsistent results and logistical constraints in using green manures and large volumes of seed meal limit their utility in nematode-control strategies. To overcome this obstacle and increase the efficacy, we have developed protocols to extract glucosinolates from mustard seed meal, concentrate the extracts, and formulate shelf-stable powdered products (Popova and Morra, 2017). Our products are derived from mustard seed from which the oil has been removed as a feedstock for biofuel. Extracts possess inherent advantages as biofumigants compared with corresponding green manures or seed meals, including (i) ease of application, (ii) decreased amount of material needed for efficacy, and (iii) greater ability to manipulate active ingredient additions to achieve efficacy. Bioactive 2-propenyl isothiocyanate is not present in the formulation but is produced enzymatically after the addition of water, thus serving to improve safety during transportation, storage, and application.

The overall goal of this research endeavor is to assess and optimize alternative eradication strategies for *G. pallida*. Another *Globodera* sp., *G. ellingtonae*, was also considered in this research because of the potential difference in hatching dynamics to that of *G. pallida* (Zasada et al., 2013). The specific objectives of the research were to quantify the activity of *B. juncea* seed meal and formulated seed meal extracts on *G. pallida* and *G. ellingtonae* hatch and reproduction in laboratory and field experiments.

Materials and Methods

Nematodes used: Because of the quarantine status of *G. pallida*, it is only possible to rear and maintain the nematode in a biosecure environment. This type of facility is available at the University of Idaho, Moscow, ID. The population of *G. pallida* used in experiments was originally obtained from an infested field in Shelley, ID. In a greenhouse, the population was reared on the susceptible potato 'Désirée' in clay pots (15.24 cm diameter) filled with a sterilized sandy loam soil and sand (2:1) mix. Plants were maintained under greenhouse conditions at a range of 10°C night-time temperature to 18°C day-time temperature, and a 16:8-h (day:night) photoperiod (standard greenhouse conditions for all experiments). Species confirmation was achieved through morphological and molecular identification (Skantar et al., 2007). After 16 wk, cysts for experimental use were recovered by extraction from soil using the Fenwick Can method (Fenwick, 1940) and picked by hand under a stereomicroscope (Leica Microsytems, Wetzlar, Germany). To estimate the number of eggs in cysts, 10 cysts were crushed with a rubber stopper, eggs were washed into a container, adjusted to a desired volume, and eggs per milliliter were determined under an inverted microscope (Leica Microsystems). Before experimental use, all cysts were incubated at 4°C for a minimum of 16 wk. Cysts were placed in sealed pouches (2.54 cm^2) made of wear-resistant nylon mesh (248.92 μm opening; McMaster-Carr, Elmhusrt, IL) and were hydrated for 3 d before use.

Globodera ellingtonae was isolated from the Oregon State University Central Oregon Agricultural Research Center, Powell Butte, OR. Inoculum for greenhouse and laboratory experiments was produced the previous field season by inoculating susceptible potato 'Russet Burbank' in the spring with *G. ellingtonae* cysts containing eggs in 22-liter pots (Grip Lip 2800; Nursery Supplies Inc., McMinnville, OR) buried in the field at Powell Butte, OR. The following spring, pots were removed from the ground, soil was emptied onto tarps, dried under shelter, thoroughly mixed, and sifted to remove rocks and large debris. The number of cysts and eggs per gram of soil was determined by extracting cysts from soil using a USDA cyst extractor (Brodie, 1996) and picked by hand under a stereomicroscope (Leica Microsystems). Cysts were then picked from a sample, counted, collected, and eggs per cyst determined as described earlier.

Mustard seed meal and seed meal extraction preparation: Mustard meal from *B. juncea* 'Pacific Gold' was obtained from

a cold press facility (University of Idaho, Moscow, ID) as previously documented (Brown et al., 1991). Mustard meal was extracted with 73% (v/v) methanol at a 1:20 v/v ratio using an end-to-end shaker at room temperature for 2 hr (Popova and Morra, 2017). Seed debris was separated by filtering and filtrates were concentrated by evaporation to remove most of the solvent. The concentrate was then freeze-dried to obtain a powder with a particle size of <45 μm with a sinigrin concentration of 381 μmol/g. The freeze-dried powder was mixed with *B. juncea* meal, potassium bicarbonate, and ascorbic acid at the ratio of 1:0.65:0.05:0.03, respectively. The concentration of sinigrin in the formulated *B. juncea* extract was 278 μmol/g.

Effect of B. juncea *seed meal rates on* G. ellingtonae *hatch and* G. pallida *reproduction:* Experiments with *G. ellingtonae* were conducted using a methodology previously used to evaluate the effects of mustard seed meals on plant-parasitic nematodes (Zasada et al., 2009). Soil (50 g), a Redmond ashy sandy loam (Fine-loamy, mixed, superactive, mesic Vitritorrandic Haploxerolls), containing cysts of *G. ellingtonae* was placed in 10 cm by 10 cm sealable plastic bags and amended with *B. juncea* seed meal at 0.1%, 0.2%, 0.3%, and 0.4% w/w (rates approximately equivalent to 2.2, 4.5, 6.7, or 8.9 t/ha); a nonamended control was included in all experiments. The corresponding approximate field rates were calculated based upon a soil bulk density of 1.45 g/cm^3 to 15 cm (bulk density for sand), which is equivalent to 2,180 t/ha soil. Treatments were replicated five times and the experiment was conducted twice. Amended soils were moistened with 5 ml water and gently mixed to ensure even distribution of water in the soil. The contents of the bags were then poured into 50-ml polystyrene tubes, packed by tapping the tube on the bench, and 1 ml water was added to the top. The tubes were incubated for 1 wk, uncapped at room temperature (approximately 25 °C). Water, approximately 2 to 3 ml, was added to the tubes when the surface of the soil dried. After incubation, the contents of the tubes were emptied onto aluminum trays and allowed to dry for 1 wk before cyst extraction. *Globodera ellingtonae* cysts were then extracted from the entirety of the sample using a USDA cyst extractor. Percentage hatch of recovered *G. ellingtonae* eggs was determined following methods described by Kroese et al. (2011). Briefly, cysts containing approximately 311 eggs/cysts were placed in 10% PRD collected from potato 'Désirée' in individual wells of a 96-well plate for 1 wk, a period of time determined to result in most of the hatch for *G. ellingtonae* (Zasada et al., 2013). Potato root diffusate (PRD) was collected from 5-wk-old potato 'Désirée' plants grown from tissue culture in 16-cm clay pots. Diffusate was collected by pouring 100 ml distilled water through the pots and the resulting diffusates were filter sterilized using 0.45 and 0.22 μm bottle-top-vacuum filters (Corning Incorporated, Corning, NY), respectively. At 1 wk, the number of hatched J2 were counted, and then the cysts were removed from the wells, cut open using a scalpel, and the number of eggs remaining determined. Percentage hatch was calculated as number of hatched J2/(number of hatched J2 + number of unhatched eggs) × 100.

For *G. pallida* reproduction assays, cysts were exposed to *B. juncea* seed meal at rates equivalent to 0, 2.2, or 8.9 t/ha in a sealed magenta GA-7 tissue culture vessel (Sigma Aldrich, St. Louis, MO). Autoclaved gravel (Moscow Building Supply, Moscow, ID), 1 cm in depth, was added to the bottom of the vessel followed by 240 g of an autoclaved 2:1 ratio of silica sand (Lane Mountain Company, Valley, WA) mixed with Prosser-series silt loam soil (WSU-IAREC, Prosser, WA). Ten cysts (containing an average of 300 eggs/cyst for an inoculation density of 5 eggs/g soil) were hydrated for 3 d and then placed in sealed 2.54-cm^2 pouches made of wear-resistant nylon mesh with 248.92 μm openings (McMaster-Carr, Elmhurst, IL). The bags containing the cysts were placed on the soil, a band of *B. juncea* seed meal at the appropriate rate was placed on the soil surface over the bag, and an additional 120 g of soil was added to cover the cyst bags. Nonamended controls received only the additional 120 g of soil. To each vessel, 50 ml of sterile deionized water was added and the magenta vessel was sealed with a lid. Five replicates of each treatment were arranged in a complete randomized design and incubated at 20°C for a period of 14 d. The experiment was conducted twice. After a 2-wk incubation, *G. pallida* mortality was assessed by removing the bags from the vessel and transferring the cyst bags to a plant bioassay. The bioassay was conducted using 6-wk-old potato 'Desirée' tissue culture plantlets planted in 10-cm clay pots (Washington Pottery Company, Kent, WA). Half the pot was filled with autoclaved 2:1 sand/Prosser soil, the cyst bag placed in the pot followed by the tissue culture plantlet, and the remaining portion of the pot filled with soil for a total of 600 g/pot. Plants were watered daily for the duration of the bioassay and fertilized using 20N–20P–20K all-purpose fertilizer (J.R. Peters Inc., Allentown, PA) three times per week as per label instructions. After 16 wk, the bioassay was terminated. Cysts were extracted from the soil using the Fenwick can method and the number of cysts per pot determined.

Impact of mustard seed meal on egg number and viability of G. ellingtonae *in a field environment:* Field trials were carried out in 2013, 2014, and 2015 at the Oregon State University Central Oregon Agricultural Research Center, Powell Butte, OR. The soil at Powell Butte, OR, is a Redmond ashy sandy loam and the climate is warm-summer Mediterranean with an average annual rainfall of 27 cm. The areas where the field trials were conducted were planted to potato inoculated with *G. ellingtonae* cysts the previous year ensuring the presence of a cyst population. Before experiment establishment, the areas were deep-ripped (30 to 35 cm) and rotovated. The same experimental design was implemented in all years, including the establishment of 3- by 1.8-m plots separated by a 1-m buffer between plots. The treatments were arranged in a randomized block design and replicated six times. In

2013, the following treatments were evaluated: (i) oat cover crop, (ii) *B. juncea* seed meal at 2.2 t/ha, and (iii) *B. juncea* seed meal at 8.9 t/ha. In 2014, the treatments were (i) bare ground, (ii) oat cover crop, (iii) *B. juncea* seed meal at 2.2 t/ha, and (iv) *B. juncea* seed meal at 8.9 t/ha. In 2015, *B. juncea* seed meal at 8.9 t/ha was compared with a bare ground control. The application of *B. juncea* seed meal involved spreading the meal on the soil surface and incorporating to an approximate depth of 10 cm with a rototiller. Both of the controls were also rototilled with oats being broadcast seeded at a rate of 78 kg/ha and lightly raked into the soil. Immediately after the experiments were established, approximately 102.8 m^3 of water was applied on the area with overhead sprinklers.

Two methods were used to assess treatment effects on *G. ellingtonae*. First, in all years, the change in egg numbers in field soil was determined by collecting soil samples before treatment and at the end of the season. Soil samples, 10 per plot, were collected using a 2.5-cm-diam. probe to a depth of approximately 15 cm and combined. In the laboratory, soil was air-dried for 1 wk, cysts were extracted from 500 g dried soil and picked with the use of a stereomicroscope as described earlier, and egg number determined as described earlier. In 2014 and 2015, a second approach was used in which packets containing *G. ellingtonae* cysts in soil were prepared as described earlier for *G. pallida*. In 2014, packets contained ~50 cysts with a mean of 307 ± 34 eggs per cyst, and in 2015, packets contained ~75 cysts with a mean of 311 ± 26 eggs per cyst ($n = 5$). Bags were buried approximately 15 cm deep immediately after treatment application but before irrigation occurred. Two weeks later, the bags were retrieved. To extract cysts, the packets were cut open and all contents placed in a 2-liter beaker followed by vigorous mixing with ~1 liter of water. The sediment was allowed to settle for 15 s before pouring the overlying liquid through nested 250- and 25-µm sieves. The sediment was suspended and the suspension decanted two more times, each time taking care to flush out cysts clinging to the sides of the beaker. Cysts were washed onto filter paper, dried, and stored at room temperature until further processing. Cysts from each sample were collected and eggs per cyst enumerated as described earlier.

Effect of B. juncea *extract on hatch of* G. pallida *and* G. ellingtonae *and reproduction of* G. pallida *in greenhouse bioassays:* Cysts of *G. pallida* were exposed to *B. juncea* extract applied at rates of 0, 1.1, 2.2, 3.3, and 4.5 t/ha in magenta vessels as described earlier. Treatments were replicated five times and the experiment was repeated. Two nylon mesh bags each containing 10 cysts (300 eggs/cyst) were placed in the magenta vessels; cysts from one set of nylon mesh bag were used for hatching assays, and the other set was used to determine subsequent reduction in *G. pallida* reproduction on potato by conducting a greenhouse bioassay. After a 2-wk exposure period, bags were recovered from vessels, and hatching assays were conducted by removing cysts from the mesh bags and releasing eggs. A 100-µl aliquot of eggs was dispensed into each of a 96-well plate and 100 µl of PRD was added to each well. Initial egg and J2 counts were determined using a stereomicroscope. Numbers of hatched J2 were determined after a 2-wk exposure to the diffusates using a stereomicroscope. Percentage hatch was calculated as described earlier.

Globodera ellingtonae was exposed to *B. juncea* extract in a greenhouse assay as described later. Soil used for the experiment was a steam-sterilized 1:1 sand:Willamette loam (Fine-silty, mixed, superactive, mesic Pachic Ultic Argixerolls). Soil was amended with the following treatments: (i) *B. juncea* seed meal at 2.2 t/ha, (ii) *B. juncea* seed meal extract at 1.1 t/ha, and (iii) nonamended control. The treatments were thoroughly mixed with 500 g dry soil. Half of the mixture was placed in a 10-cm clay pot and a cyst packet with 20 cysts of *G. ellingtonae* (prepared as described earlier for *G. pallida*) was placed on the soil surface. The remainder of the soil was added to the pot and the pot was watered with approximately 75 ml water. The pots were placed in a plastic bin containing washed sand so that the pots were buried with approximately 3 cm of pot remaining exposed. The pots were arranged in a completely randomized design on a greenhouse bench, treatments were replicated six times, and the experiment was conducted twice. The greenhouse was maintained with a 16-hr/d photoperiod; temperatures were set to 25°C during the day and 20°C at night. The experiment duration was 6 wk with pots receiving water when needed. Upon termination, the cyst packets were removed and eight cysts of similar size were recovered and placed in a PRD hatching assay as described earlier for *G. ellingtonae*.

Effect of B. juncea *seed meal extract on* G. pallida *in field microplots: Globodera pallida* populations in infested fields in Idaho are low in density, highly aggregated, and variable in both viability and in egg numbers (T. Gresham, personal communication). Increasing those populations by growing potato is not possible because of restrictions in place by USDA-APHIS and ISDA. To demonstrate the potential of *B. juncea* seed meal extracts under field conditions, greenhouse-reared *G. pallida* cysts were used. In addition, a containment protocol was developed and approved by USDA-APHIS and ISDA to prevent the possible escape of introduced cysts. The containment microplots consisted of 18.9-liter plastic buckets placed into the ground and backfilled with field soil. Cysts were contained and sealed in nylon mesh bags (as described earlier) and at the end of the growing season, cysts were retrieved, and transported to University of Idaho, Moscow, ID, to conduct egg counts and hatching assays in a USDA-APHIS approved facility.

Microplot trials were conducted in Shelly, ID, in 2015 and 2016. Microplots were prepared by drilling four 1-cm holes in the bottom of a bucket (18.9 liter) and sealing nylon mesh (same as used for cyst bags) over each hole

with silicon caulking. To prevent any possible escape of *G. pallida* into the surrounding field soil, each bucket was then placed inside of another bucket of the same size that had not been drilled, and the unit was placed into holes in the soil created by a backhoe. *Brassica juncea* seed meal extract at a rate of 4.5 t/ha (28.7 g extract added to each bucket) was compared with a nonamended control by banding the seed meal extract on top of the soil. Treatments were replicated six times and arranged in a randomized block design in both experiments. Each bucket was filled with 18.5 kg of sandy loam field soil. Nylon mesh bags (20 cysts/bag and 8 bags/bucket to obtain a rate of 2.5 eggs/g soil) prepared as described earlier were then placed 15 cm below the soil surface and covered with 10 cm of soil. The nylon mesh bags were attached to a stake for ease of retrieval. The *B. juncea* seed meal extract treatment was then banded over the soil surface and then covered by an additional 5-cm of soil. After treatment, 500 ml of water was added to each bucket to activate hydrolysis of sinigrin. To simulate a closed system, the buckets were tightly sealed with a lid for the duration of the experiment. To assess treatment effects, cyst bags were removed from the microplots after 10 and 6 wk in 2015 and 2016, respectively, and *G. pallida* egg numbers and hatching assays were conducted as described earlier.

Data analysis: Data were analyzed by analysis of variance using the General Linear Model statement in Statistical Analysis Software (SAS), SAS Institute Inc., Cary, NC. To meet analysis of variance assumptions, square root or arcsine transformations were used to ensure a normal distribution and constant variation of the count and hatching data, respectively. Statistically significant differences among treatments were computed by the Tukey–Kramer multiple comparison test with significance level at $P < 0.05$.

Results

Effect of B. juncea *seed meal rates on* G. ellingtonae *hatch and* G. pallida *reproduction in laboratory and greenhouse assays:* In laboratory bioassays, a rate of 8.9 t/ha *B. juncea* meal almost completely inhibited hatch of *G. ellingtonae* eggs (Table 1). In trial 1, this reduction was similar to that observed with lower *B. juncea* seed meal rates, 4.5 and 6.7 t/ha, but in trial 2, the reduction in hatch by the highest seed meal rate was significantly lower than any of the other treatments. In trial 2, the lowest rate of *B. juncea* seed meal tested, 2.2 t/ha, resulted in similar hatch of *G. ellingtonae* eggs as in trial 1, but a 40% lower egg hatch compared to the nonamended control. In the *G. pallida* assays, exposure to *B. juncea* seed meal for 2 wk eliminated subsequent *G. pallida* reproduction on potato at both rates tested (Table 2). In both trials, no significant difference in reproduction was observed whether the meal was applied at a rate of 2.2 or 8.9 t/ha (Table 2). Both rates of *B. juncea* seed meal resulted in a 99.9% to 100% reduction in *G. pallida* cysts per pot.

Table 1. Effect of *Brassica juncea* seed meal on hatch of *Globodera ellingtonae* eggs.

	Trial 1	Trial 2
Rate (t/ha)	Hatch (%)[a]	
Nonamended	56.3 ± 3.5 a[b]	56.7 ± 4.1 a
2.2	43.7 ± 3.1 a	33.9 ± 4.2 b
4.5	7.9 ± 5.2 b	32.9 ± 5.0 b
6.7	8.8 ± 2.1 b	14.2 ± 3.3 c
8.9	0.1 ± 0.2 b	0.1 ± 0.1 d

[a] Percentage hatch was determined using a potato root diffusate hatching assay (Kroese et al., 2011).
[b] Values ± standard errors are the average of six replicates. Values within a column followed by a common letter are not significantly different according to the Tukey–Kramer test ($P < 0.05$).

Application of B. juncea *seed meal for* G. ellingtonae *suppression in a field environment:* Across all 3 years, there was no reduction in numbers of *G. ellingtonae* eggs after the application of *B. juncea* seed meal at either rate, compared with the nonamended bare ground or oat controls (Table 3). When averaged across treatments, there were 77.5%, 51.9%, and 54.4% reductions in egg densities over the growing season in 2013, 2014, and 2015, respectively (Table 3). Deleterious effects of *B. juncea* seed meal on *G. ellingtonae* hatch rate were observed at all rates tested (Table 3). When soil was treated with *B. juncea* at 8.9 t/ha, egg hatch was almost completely inhibited in 2014 and reduced by 83% compared with the bare ground control when repeated in 2015. This percentage hatch was significantly less than egg hatch of eggs recovered from the nonamended control or from *B. juncea* applied at 2.2 t/ha treatment. When applied at a lower rate (2.2 t/ha), *B. juncea* meal resulted in 50% fewer eggs hatching compared with the nonamended control (Table 3).

Effect of B. juncea *seed meal and extract on hatch of* G. ellingtonae *and hatch and reproduction of* G. pallida *in greenhouse bioassays:* In experiments with *G. ellingtonae*, a similar trend was observed across trials (Table 4). Amendment of soil with *B. juncea* seed meal or extract resulted in almost complete inhibition of egg hatch compared with the nonamended control, even though the rate of application of the meal extract was 50% less than that of the seed meal. In experiments with *G. pallida*, *B. juncea* seed meal extract significantly reduced *G. pallida* hatch by 97.0% to 99.7% at all rates tested compared with the nonamended control (Table 5). No

Table 2. Effect of a 2-wk exposure to *Brassica juncea* seed meal on *Globodera pallida* reproduction on potato 'Désirée'.

	Trial 1	Trial 2
Rate (t/ha)	Cysts per pot	
Nonamended	303.0 ± 82.0 a[a]	234.0 ± 44.0 a
2.2	0.0 ± 0.0 b	0.0 ± 0.0 b
8.9	0.3 ± 0.3 b	0.0 ± 0.0 b

[a] Values ± standard errors are the average of five replicates. Values within a column followed by a common letter are not significantly different according to the Tukey–Kramer test ($P < 0.05$).

TABLE 3. Percentage change in *Globodera ellingtonae* egg densities and percentage hatch of eggs exposed to *Brassica juncea* (*Bj*) seed meal in the field at Powell Butte, OR.

	2013	2014		2015	
Treatment	Reduction in egg number (%)[a]	Hatch (%)	Reduction in egg numbers (%)	Hatch (%)	Reduction in egg numbers (%)
Bare ground	ND[b]	74.3 ± 3.2 a[c]	41.8 ± 12.8	65.2 ± 5.3 a	52.3 ± 7.4
Oats	68.1 ± 3.0	ND	58.3 ± 10.8	ND	ND
Bj seed meal 2.2 t/ha	86.7 ± 5.4	37.6 ± 4.9 b	59.4 ± 8.7	ND	ND
Bj seed meal 8.9 t/ha	77.7 ± 7.5	0.1 ± 0.1 c	48.3 ± 8.3	11.2 ± 9.1 b	56.1 ± 10.1

[a] Reduction in egg numbers based on the initial egg population for each plot.
[b] Not determined (ND).
[c] Values ± standard errors are the average of three replicates. Values within a column followed by a common letter are not significantly different according to the Tukey–Kramer test ($P < 0.05$).

significant difference was found in the percentage hatch rate across *B. juncea* seed meal extract rates. Subsequent to the exposure of *G. pallida* to different rates of *B. juncea* seed meal, *G. pallida* reproduction on potato grown under greenhouse conditions was significantly less than that of the nonamended control, as evidenced by decreased cysts per pot (Table 5). A 2-wk exposure to all rates of seed meal extract tested significantly reduced *G. pallida* reproduction on potato in a 16-wk greenhouse trial compared with the nonamended control.

Effect of B. juncea *seed meal extract on* G. pallida *in field microplots:* In 2015, the number of eggs remaining in cysts 10 wk after the application of *B. juncea* seed meal extract was significantly less (73%) than that in the nonamended control (Table 6). In 2016, the number of eggs remaining in cysts 6 wk after treatment exposure was not significantly altered whether or not *B. juncea* meal extract was amended to soil (Table 6). However, the percentage hatch of the remaining population of eggs was 90% lower 6 wk post seed meal extract amendment than the nonamended control (Table 6).

DISCUSSION

The combined findings from laboratory, greenhouse, and field experiments evaluating *B. juncea* seed meal and seed meal extract demonstrate the utility of *B. juncea*–derived amendments as a valuable component in an integrated eradication strategy for *G. pallida* and other *Globodera* spp. Our results reveal that soil amendment with a *B. juncea* seed meal extract formulated from *B. juncea* seed meal reduced hatch and egg densities of both *G. pallida* and *G. ellingtonae*, and eliminated reproduction of *G. pallida*. Across laboratory and greenhouse assays, *B. juncea* seed meal was evaluated at rates ranging from 2.2 to 8.9 t/ha (rates equivalent to approximately 0.1% to 0.4% of soil weight). Because seed meal was tested in an open assay system (without a plastic film or mulching) for *G. ellingtonae*, *B. juncea* seed meal rates greater than 4.5 t/ha were required for consistent reduction in egg hatch of this nematode. When a closed system was used for *G. pallida*, the lowest rate of *B. juncea* seed meal tested, 2.2 t/ha, was effective. The rates of *B. juncea* seed meal evaluated in these experiments are within the range of rates demonstrated to suppress other plant-parasitic nematodes in other studies. In a side-by-side comparison of *Pratylenchus penetrans* and *Meloidogyne incognita*, *B. juncea* seed meal applied at 0.1% of soil weight resulted in 97% nematode reduction of both species (Zasada et al., 2009). On sweet corn, *P. penetrans* was suppressed when *B. juncea* seed meal was applied at a rate equivalent to 0.025% (Yu et al., 2007). A higher rate of *B. juncea* seed meal, 0.3% of soil weight, was required to suppress population densities of *P. penetrans* in apple roots (Mazzola and Zhao, 2010); however, this same rate did not suppress *P. penetrans* densities in soil. Brassicaceous residues incorporated into soil reduced both viability of *G. pallida* encysted eggs and increased the mortality of hatched J2 (Pinto et al., 1998; Buskov et al., 2002; Serra et al., 2002; Lord et al., 2011).

In comparisons of brassicaceous seed meal combinations with preplant 1,3-dichloropropene or chloropicrin soil fumigation in apple replant disease, seed meal–amended soils were resistant to reinfestation by *P. penetrans* and *Pythium* spp. and corresponded with better tree performance (Mazzola et al., 2015). Amendment of soil with *B. juncea* seed meal resulted in 100% suppression of both *Pratylenchus* and *Meloidogyne* (Zasada et al., 2009) However, the effective seed meal rate (10.0% dry w/w) (Zasada et al., 2009) would be impractical for potato production and may contribute to phytotoxicity. Other advantages of effective activity at low rates, which the formulated seed meal extract provides, include compatibility with organic systems and no loss of crop profit from planting to a green manure crop for biofumigation purposes.

TABLE 4. Hatch of *Globodera ellingtonae* after exposure to *Brassica juncea* seed meal and seed meal extract in a greenhouse trial.

	Trial 1	Trial 2
Treatment	Hatch (%)[a]	
Seed meal 2.2 t/ha	0 a[b]	0 a
Seed meal extract 1.1 t/ha	1.0 ± 1.0 a	0 a
Nonamended	73.2 ± 3.2 b	59.8 ± 4.2 b

[a] Percentage hatch was determined using a potato root diffusate hatching assay (Kroese et al., 2011).
[b] Values ± standard errors are the average of six replicates. Values within a column followed by a common letter are not significantly different according to the Tukey–Kramer test ($P < 0.05$).

TABLE 5. Percentage hatch of *Globodera pallida* eggs after a 2-wk exposure to five rates of *Brassica juncea* seed meal extract, followed by a greenhouse bioassay to assess reproduction of exposed cysts on potato 'Désirée' for 16 wk.

	Trial 1		Trial 2	
Extract rate (t/ha)	Hatch (%)	Cysts per pot	Hatch (%)	Cysts per pot
Nonamended	70.0 ± 6.5 a[a]	538.3 ± 62.6 a	9.6 ± 4.0 a	60.4 ± 26.8 a
1.1	1.0 ± 0.3 b	1.0 ± 0.8 b	0.8 ± 0.8 b	0.2 ± 0.2 b
2.2	1.8 ± 0.5 b	0.2 ± 0.2 b	0.6 ± 1.3 b	0 b
3.3	0.2 ± 0.2 b	0.0 ± 0.0 b	0.4 ± 0.5 b	0 b
4.5	0.2 ± 0.2 b	0.4 ± 0.4 b	0.2 ± 0.4 b	0 b

[a] Values ± standard errors are the average of six replicates. Values within a column followed by a common letter are not significantly different according to the Tukey–Kramer test ($P < 0.05$).

Although *B. juncea* seed meal did suppress *G. pallida* and *G. ellingtonae* egg hatch and *G. pallida* reproduction on potato, there is also the opportunity to significantly reduce application rates by using an extracted seed meal product. Our results reveal that soil amendment with a *B. juncea* seed meal extract containing 2-propenyl glucosinolate (sinigrin) formulated from *B. juncea* seed meal reduced hatch and egg densities of both *G. pallida* and *G. ellingtonae*, and eliminated reproduction of *G. pallida*. Amendment of soil with an extract prepared from *B. juncea* seed meal applied at a rate of 1.12 t/ha nearly killed all encysted eggs, as evidenced by the greatly reduced hatch and low reproduction factor.

The goal of extracting the active component of *B. juncea* seed meal is to produce an efficacious bionematicide that does not suffer from the limitations of using mustard seed meals, including (i) batch-to-batch variability, (ii) high costs associated with transportation, storage, and application of relatively large amounts of material, and (iii) unexpected and potentially negative impacts of amending soils with large amounts of organic material and nitrogen (Popova and Morra, 2017). Results clearly indicate that an extract rate as low as 1.1 t/ha (equivalent to 0.05% of soil weight) suppresses both *G. pallida* and *G. ellingtonae*.

Our results are consistent with reports that glucosinolates found in many brassicaceous species degrade through enzymatic hydrolysis into biologically active volatile products (Brown and Morra, 1997; Rosa et al., 1997; Warmington and Clarkson, 2016) known to be toxic to nematodes (Aires et al., 2009; Zasada et al., 2009; Lord et al., 2011; Brolsma et al., 2014). The most toxic glucosinolate metabolites are isothiocyanates, including 2-propenyl isothiocyanate produced in high concentrations from the primary glucosinolate in *B. juncea* seed (Popova and Morra, 2014). The nematicidal activity of *B. juncea* meal is generally attributed to the production of 2-propenyl isothiocyanate (Zasada et al., 2009; Lord et al., 2011). Isothiocyanates act as general biocides because they interact irreversibly with sulfhydryl groups in proteins, disulfide bonds, and amines, thereby deactivating a variety of metabolic pathways (Kawakishi and Kaneko, 1985; Keppler et al., 2014).

TABLE 6. *Globodera pallida* egg densities and percentage hatch of eggs exposed to *Brassica juncea* seed meal extract (4.5 t/ha) for 10 wk (2015) or 6 wk (2016) in Shelly, ID.

	2015		2016	
Treatment	Eggs per cyst	Hatch (%)	Eggs per cyst	Hatch (%)
Bare soil	299 ± 20 a[a]	ND[b]	100 ± 29	19.0 ± 2.9 a
B. juncea extract	81 ± 14 b	ND	139 ± 16	1.9 ± 2.8 b
P value	0.001	-	0.15	0.001

[a] Values ± standard errors are the average of six replicates. Values within a column followed by a common letter are not significantly different according to the Tukey–Kramer test ($P < 0.05$).
[b] Not determined (ND).

The experimental venue in which *G. pallida* was exposed to *B. juncea* seed meal and seed meal extract was different from that used for *G. ellingtonae*. In all of the *G. pallida* laboratory, greenhouse, and field experiments, soil amended with *B. juncea* seed meal or seed meal extract was moistened and then the system was closed. In *G. ellingtonae* experiments, the soil was moistened and the system was left open; thus, a higher rate of mustard seed meal was needed for suppression of this nematode. The effect of the assay system being open or closed on nematode suppression became evident when evaluating lower rates of *B. juncea* seed meal. In the closed system, the lowest rate tested, 2.2 t/ha, resulted in similar suppression of *G. pallida* as the highest rate tested, 8.9 t/ha. This same trend was not observed for *G. ellingtonae*, with the *B. juncea* seed meal rate of 2.2 t/ha never resulting in similar suppression of *G. ellingtonae* as 8.9 t/ha. The difference in results is likely explained by the chemical characteristics of the active ingredient produced during glucosinolate hydrolysis. 2-Propenyl isothiocyanate has a vapor pressure of approximately 500 Pa at 25°C, thus making it a highly volatile compound (Lim and Tung, 1997). Open containers resulted in volatile losses of isothiocyanate that caused a decrease in fumigant concentration and correspondingly weaker nematicidal activity.

In most of our experiments, egg hatch was used as an indicator of treatment efficacy. In some experiments, reduction in *Globodera* spp. egg numbers and the reproduction potential of *G. pallida* were also assessed. Although a reduction in egg numbers was not consistently observed, the biological processes of *Globodera* spp. were disrupted with reduced hatch and viability, as assessed by reproduction of exposed eggs. Because *G. ellingtonae* behaves more similar to *G. rostochiensis* than *G. pallida*,

based upon hatching assays and similarity in parasitism on susceptible and resistant potato varieties (Zasada et al., 2013), we included these two species in the experiments to determine if there might be differential effects of the seed meal and extract. *Globodera pallida* is reported to have a much slower hatch than *G. rostochiensis* (Whitehead, 1992). Part of this difference in hatch may be because *G. pallida* egg permeability may be more resistant to changes in the environment to ensure survival. Therefore, it was unknown whether *Globodera* spp., with different hatching dynamics, would respond similarly to the release of compounds from *B. juncea* seed meal and extract. The response in hatch suppression was similar for *G. pallida* and *G. ellingtonae*, demonstrating the widespread applicability of this control method.

As previously mentioned, the primary sinigrin hydrolysis product thought to be responsible for the observed activity is 2-propenyl isothiocyanate (Borek et al., 1994). The half-life of 2-propenyl isothiocyanate in different soils has been reported to range from 20 to 60 hr (Borek et al., 1995). Although this is a relatively short half-life for a nematicide, in vitro experiments have shown that a median lethal time of only 1.85 hr is required to suppress *G. pallida* hatch when exposed to 0.002% 2-propenyl isothiocyanate (Brolsma et al., 2014). Direct comparisons of this concentration to those used in our experiments is problematic, given the difference in bioassay conditions; however, it is clear that short exposure times to 2-propenyl isothiocyanate potentially disrupt *G. pallida* reproduction.

Although we have shown that seed meal extracts have advantages and potential utility in controlling two different nematodes, application of lethal concentrations of *B. juncea* seed meal extract under field conditions warrants further investigation. For example, field microplots in Shelley, Idaho, demonstrated variability in the level of PCN control, variability that may be related to experimental conditions in which lids were placed on the buckets to provide a closed system. However, applying a plastic mulch may be a more efficient method to trap the volatile active ingredient. Other factors that may contribute to variability include soil moisture and temperature, which have been implicated in the movement of 2-propenyl isothiocyanate throughout the soil profile, as well as microbial activity (Snyder et al., 2009). In addition to identifying critical field variables that inhibit practical application, improvements in extract formulation will improve efficacy. Our current efforts to increase sinigrin concentrations, and thus active ingredient production from the extracts, will promote practical application by decreasing the volume of material required for pest suppression. Activities are also under way to scale up and commercialize the extraction procedure. To ensure economic success, we have developed a processing protocol focused on zero waste, recovery and reuse, and multiple high-value products including the described natural nematicide. Future assessments of *B. juncea* seed meal extract as an effective and consistent soil amendment for *Globodera* spp. control will thus need to take into consideration environmental conditions and improved product formulations.

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