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Suppressive Effect of *Brassica juncea* on the Root-Lesion Nematode (*Pratylenchus penetrans*)

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Abstract: Incorporation of sulfur-rich crucifer tissues into soil is known to suppress a variety of soil-borne plant pathogens and pests. The potentials of using *Brassica juncea* as green manure to kill the root lesion nematode *Pratylenchus penetrans* in soil and to decrease damages to subsequent crops were assessed in field and pot experiments. In the first trial, green manures containing *B. juncea* were grown and incorporated during spring to summer 2009. Japanese radish was then cultivated in each plot. In the second trial in spring 2010, green manure was grown and incorporated during summer to autumn 2009, and greater burdock was cultivated in pots containing soil sampled from each plot. Neither trial showed clear effects on nematode populations in the soil. However, in the first trial, Japanese radish grown following a *B. juncea* breeding line with a high content of sinigrin had a lower root lesion index and a higher number of marketable taproots than grown in the fallow soil. In the second trail, greater burdock cultivated in pots following incorporation of *B. juncea* had a lower root lesion index with the incorporation of white mustard, which is widely used as a landscape plant. These findings suggest that *B. juncea* used as green manure can potentially decrease damage to subsequent crops caused by the root-lesion nematode, although it had no positive effect on decreasing populations of the root-lesion nematode in the soil.

Key words: Biofumigation, green manure, *Brassica juncea*, *Pratylenchus penetrans*, root-lesion nematode, soil.

1. Introduction

Pratylenchus penetrans (Cobb) is the most common root-lesion nematode in Hokkaido, the north island of Japan [1], and is a destructive species in a wide range of crops. In potato and adzuki bean, yield losses of 14% to 20%, respectively, due to this nematode have been reported in the Tokachi area of Hokkaido [2]. Control of the pest currently relies on the use of chemical fumigants, which are increasingly becoming a nonviable option because of their economic and environmental costs. Antagonistic crops such as marigold (*Tagetes* spp.) are known to suppress plant parasitic nematodes, such as root-lesion and root-knot nematodes [3]. However, because the above crops have poor vigor at early growth stages, weed control is necessary. Thus, lopsided oat (*Avena strigosa* Schreb.) has been widely used as an antagonistic plant to control root-lesion nematode in Hokkaido because of its high yield, rich root system and good early vigor [4].

Crucifer tissues have long been known to produce biocidal effects following their addition to soil, in a process known as "biofumigation" [5]. Brassica crops contain glucosinolates, which are sulfur-containing, stable, and non-toxic compounds. There are over 100 different glucosinolates [6], which are considered to have little biological activity themselves but have been associated with plant defense [7]. After tissue damage, these molecules are cleaved by a thioglucosidase, producing many products including

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isothiocyanates, nitriles, and thiocyanates. Isothiocyanates are biologically active, disrupting cellular components by denaturing the protein structure [8]. Incorporation of crucifer residues into various soil types has been found to reduce the activity of several fungal pathogens, including the fungi *Aphanomyces* [9] and *Gaeumannomyces* [10]. Similarly, crucifer tissues may have the potential to control a variety of invertebrate pests, including nematodes [11] and insects [12]. A reduction in the number of many soil-borne plant parasitic nematodes with incorporation of *Brassica* tissues or the products of glucosinolates has been observed, including in such species as *Meloidogyne incognita*, *M. javanica* [13], *Heterodera schachtii* [14], and *Globodera rostochiensis* [15]. Green manuring with tissues of *Brassica* spp. was found to suppress populations of the root-lesion nematode [16, 17]. Although *B. juncea* is a good host [18], allowing nematodes to feed and reproduce during its cultivation, a decrease in nematode numbers might not be expected.

We previously showed a significant pathogen reduction using *Brassica* species as green manure to control soil-borne plant disease [19, 20]. Here, we examined the nematicidal impact of soil containing tissues of oriental mustard. The study objective was to evaluate the potential of *B. juncea* to control *P. penetrans* under field conditions.

2. Materials and Methods

2.1 Experiment 1

The field experiment was conducted at the Hokkaido research station of Snow Brand Seed Co., Ltd., Naganuma town (4255′13.9″ N, 14143′11.8″ E), Hokkaido, Japan. Plot size was 2 m × 2 m and the experiment was arranged in a randomized complete block design with four replications. Nine soil cores 10-20 cm deep were collected on 12 May 2009 from each plot and mixed to determine initial nematode densities. Nematodes were extracted from the samples using the Baermann method, from 20 g soil for 72 h at 20 °C. Green manure crops were sown on 15 May 2009. Lopsided oat (*A. strigosa* Schreb., 150 kg/ha; “Hayoats”) and oriental mustard breeding lines (*B. juncea* (L.) Czern., 10 kg/ha; “Y-010” and “17-10”) were sown by hand in each plot, with 30 cm spacing between rows, and raked into the soil. Plots in which weeds were eradicated by hand were used as a control treatment (fallow). Eighty kilograms per hectare of NPK fertilizer were applied to green manure crops and the control treatment at each sowing time. Soil samples were collected in the same way as for sampling the initial nematode population, after green manure crops were incorporated into the soil with a rotovator twice on 30 June 2009. Green manures were also sampled to estimate total biomass production. Samples were collected from each plot by clipping all plants at soil level, placing all foliage in a container, and weighing the material. Random subsamples of fresh biomass from each plot were weighed in the laboratory, dried in oven at 75 °C for 72 h, and reweighed to calculate moisture content.

Oriental mustard crops were sampled to determine glucosinolate content. From both oriental mustard crop plots within each block, a sample of five plants was carefully collected by clipping plants at soil level, and kept cold during prompt transit to a freezer for storage until freeze drying. Freeze-dried plants were ground using a mill, and the samples were analyzed with high performance liquid chromatography (HPLC). Crude glucosinolates from 100 mg of freeze-dried shoot tissue were extracted with 3 mL of boiling ultrapure water in a waterbath at 100 °C for 1 h to inactivate the endo-myrosinase. The mixture was centrifuged (3,000 rpm, 30 min) and the resulting supernatant collected. This crude extract was applied to a Minisart RC15 filter (0.2 μm). HPLC was performed using a Prominence system (Shimadzu, Kyoto, Japan). The eluate (5 μL) was injected into a 4.6 mm × 250 mm i.d. Waters Atlantis dc18 (5 μm) column. The measurement wavelength was set at 230 nm using an SPD-20A ultraviolet/visible detector

(Shimadzu). The HPLC was operated in constant flow mode at 1.0 mL min^{-1}, at an oven temperature of 35 °C. The glucosinolates were quantified using external standards (sinigrin) prepared in 0.1 M ammonium acetate.

The final nematode populations in the soil were sampled from each plot on 3 August 2009. Japanese radish (*Raphanus sativus* L., "Bantyukitaichi") was sown in all plots at a 60 cm spacing between rows and 25 cm between plants on 5 August 2009. Fifty kilograms per hectare of NPK fertilizer were applied for Japanese radish crop and managed according to standard practices. Taproots were sampled from 10 plants randomly selected from the middle part of each plot on 12 October 2009. All harvested roots were rinsed in water, weighed and rated for root-lesion severity. The severity of root-lesion symptoms was recorded on a 0-to-4 ordinal scale [21]. The root-lesion index was represented as a proportion, calculated as the sum of (number of tap roots in a category × a category)/4 × (total number of scored tap roots). Marketable tap roots were defined as the percentage of roots with a root-lesion index of 0 to 2, which is an acceptable level for food crops.

2.2 Experiment 2

After winter wheat was harvested, a field experiment was conducted at Bihoro town Hokkaido, Japan. The plot size was 6 m × 6 m and the experiment was arranged in a randomized complete block design with four replications. Ten soil cores 10-20 cm deep were collected from each plot on 12 August 2009 and mixed to determine initial nematode densities. Nematodes were extracted from the samples using the Baermann method from 20 g soil for 72 h at 20 °C. Green manure crops were sown on 13 August 2009. Lopsided oat (*A. strigosa*, 150 kg/ha; "Hayoats"), an oriental mustard breeding line (*B. juncea*, 10 kg/ha; "Y-010"), and white mustard (*Sinapis alba* L., 20 kg/ha; "Kikarashi") were sown by hand in each plot and raked into the soil. Plots with fallen seeds of wheat were used as a control treatment. Eighty kilograms per hectare of NPK fertilizer were applied to green manure crops and the control treatment at each sowing time. Soil samples were collected in the same way as for sampling the initial nematode population, and then green manure crops were incorporated into the soil with a chopper and plough on 16 October 2009. Estimations of the biomass of green manure crops and glucosinolates analysis of *Brassica* crops were conducted as in experiment 1.

The final nematode populations in soil were sampled from each plot on 18 May 2010. These final soil samples from the field plots were moved into a greenhouse at the Hokkaido research station of Snow Brand Seed Co., Ltd., Naganuma town in Hokkaido. A total of 10 kg of soil from each plot was mixed with 10 g of fertilizer (N: 15%, P: 15%, K: 15%) and partitioned into four at 1/5,000 a Wagner pots (each of 2.5 kg) and 15 greater burdock seeds (*Arctium lappa* L., "Tegaru") were planted per pot. The burdock seedlings were thinned to five per pot. After 60 days of cultivation, taproots were sampled from each pot. All harvested roots were rinsed in water and rated for root-lesion severity. The severity of root-lesion symptoms was recorded on a 0-to-4 ordinal scale: 0, no symptoms; 1, very few brown spots; 2, brown spots recognized on root surface; 3, brown spots covering half of root surface; 4, brown spots all over root surface. The severity was examined from each taproot in the pots and the root-lesion index was represented as a proportion for each pot, calculated as the sum of (number of tap roots in a category × a category)/4 × (total number of scored tap roots).

2.3 Data Analysis

All statistical analyses were carried out using the R statistical environment for statistical computing and graphics, version 2.11.1. [22]. The green manure yields and sinigrin concentrations were analyzed using analysis of variance (ANOVA). The means of sinigrin concentrations were compared using a *t*-test. The population of nematodes in the soil was analyzed using a generalized linear mixed model (GLMM) with Poisson distribution [23]. The severity of root lesions on Japanese radish and greater burdock was also recorded on a category basis as ordinal data using multiple scales for the experiment. Because the root-lesion index of Japanese radish tap roots or greater burdock and the rate of marketable Japanese radish were proportional data (from 0 to 1) and characterized by a binomial distribution, a GLMM was used for these data [24, 25]. Treatment and block were treated as fixed and random effects, respectively. Fitting a GLMM to the data was done using the Laplace approximation with the lme4 package [26]. The reproduction ratio of nematode population in soil was analyzed with a linear mixed model [23] with the nlme package [27]. Significant differences among these treatments were separated using Tukey's all-pairwise comparison with the multcomp package [28].

3. Results

3.1 Experiment 1

The oriental mustard in the field experiment yielded a slightly higher biomass than the lopsided oat. Sinigrin content differed significantly between oriental mustard crops: the content of breeding line "Y-010" was higher than that of "17-10" (Table 1).

The *B. juncea* "17-10" sown in spring increased population densities of root-lesion nematodes, but breeding line "Y-010" slightly decreased the population compared with the initial density. The efficacy of mustard incorporation in reducing nematode populations in the soil was lower than in the fallow plots (Table 2). The population of nematodes in each soil decreased when Japanese radish was cultivated, and there was no trend toward increased population densities of root-lesion nematodes in mustard plots.

The root lesion index of Japanese radish in the oriental mustard "Y-010" plot was significantly lower than that in the fallow plot (Table 3). Furthermore, the proportion of marketable tap roots in the oriental mustard "Y-010" was higher than in the fallow plot.

3.2 Experiment 2

Although a significant difference was not observed, the dry biomass of oriental mustard "Y-010" averaged 4.85 ± 0.32 t/ha and was slightly lower compared to other green manure crops (Table 4). Oriental mustard contained 20.1 ± 2.3 μmol/g of sinigrin, which was higher than that of white mustard.

Table 1 Dry weight biomasses from green manure and sinigrin concentrations of oriental mustard in experiment 1.

Plant	Breeding line and Cultivar	Mean biomass (metric tons/ha)	Sinigrin concentration (μmol g^{-1})
Oriental mustard	Y-010	3.07 ± 0.07	16.4 ± 1.0a
	17-10	3.54 ± 0.28	12.5 ± 0.4b
Lopsided oat	Hayoats	2.95 ± 0.13	-

Each value is the average and standard error from four observations. Values followed by the same letter within a column are not significantly different ($P < 0.05$) according to *t* test.

Table 2 Fluctuation in *Pratylenchus penetrans* soil densities during spring cultivation in experiment 1.

Plant	Breeding line and Cultivar	Nematode population in soil (no./20 g)			
		P_i	P_m	P_f	P_f/P_i
Oriental mustard	Y-010	134 a	117 b	56 a	0.5 a
	17-10	97 b	230 a	52 a	0.6 a
Lopsided oat	Hayoats	115 ab	67 c	19 c	0.2 b
Fallow	-	130 a	80 c	37 b	0.3 ab

Each value is the average from four observations. Values followed by the same letter within a column are not significantly different ($P < 0.05$) according to Tukey's all-pairwise comparison test. P_i is the initial nematode population from samples collected on May 12; *P*m is the midseason nematode population from samples collected on June 30; P_f is the final nematode population from samples collected on August 3; P_f/P_i is reproduction ratio.

Table 3 Damage to Japanese radish after spring cultivation of green manures in a *Pratylenchus penetrans* infested field in experiment 1.

Preceding plant		Damage to Japanese radish	
Plant	Breeding line and cultivar	Root lesion index	Marketable taproots (%)
Oriental mustard	Y-010	65.6 ± 4.1 bc	35.0 ± 12.6 b
	17-10	71.3 ± 5.8 ab	22.5 ± 13.1 bc
Lopsided oat	Hayoats	56.9 ± 1.6 c	72.5 ± 6.3 a
Fallow	-	83.1 ± 4.1 a	7.5 ± 6.3 c

Each value is the average from four observations and standard error. Values followed by the same letter within a column are not significantly different ($P < 0.05$) according to Tukey's all-pairwise comparison test. Root-lesion index = [(ratings × no. of plants involved)/(total no. of plants examined × 4)] × 100. Marketable taproots indicate a root lesion degree of two or less.

Table 4 Dry weight biomasses of green manures and sinigrin concentrations of oriental mustard in experiment 2.

Plant	Breeding line and Cultivar	Mean biomass (metric tons/ha)	Sinigrin concentration (μmol g^{-1})
Oriental mustard	Y-010	4.85 ± 0.32	20.1 ± 2.3 a
White mustard	Kikarashi	5.50 ± 0.26	0.4 ± 0.3 b
Lopsided oat	Hayoats	5.19 ± 0.14	-

Each value is the average and standard error from four observations. Values followed by the same letter within a column are not significantly different ($P < 0.05$) according to t test.

Table 5 Fluctuation in *Pratylenchus penetrans* soil densities during summer cultivation in experiment 2 and damage to greater burdock in the final soil samples.

Plant	Breeding line and Cultivar	Nematode population in soil (no./20 g)				Damage to greater burdock
		P_i	Pm	P_f	P_f/P_i	Root lesion index
Oriental mustard	Y-010	23 a	15 a	19 a	0.8 a	87.3 ± 3.0 b
White mustard	Kikarashi	14 b	20 a	13 ab	1.1 a	94.6 ± 1.6 a
Lopsided oat	Hayoats	18 a	8 b	11 b	0.5 a	75.4 ± 3.2 c
Fallow	-	18 a	7 b	13 ab	0.7 a	88.8 ± 4.5 b

Each value is the average from four observations. Values followed by the same letter within a column are not significantly different ($P < 0.05$) according to Tukey's all-pairwise comparison test. P_i is the initial nematode population from samples collected on August 12; Pm is the midseason nematode population from samples collected on October 16; P_f is the final nematode population from samples collected on May 18, 2010; P_f/P_i is reproduction ratio. Root-lesion index = [(ratings × no. of plants involved)/(total no. of plants examined × 4)] × 100 and the value is the average and standard error from four observations.

There was little difference in nematode populations between mustard-treated plots and fallow plots. Initial nematode populations ranged from 14 to 23 per 20 g of soil (Table 5). Approximately 2 months later, nematode populations were reduced to about 10 after incorporation of green manure, except for the white mustard plot where the final nematode populations ranged from 11 to 19 per 20 g of soil (including fallow).

The root lesion index of greater burdock in the mustard "Y-010" plots was significantly lower than that in the white mustard plot, but there was no significant difference in the index between mustard and fallow plots (Table 5).

4. Discussion

Our study shows that oriental mustard used for green manure decreased damage intensity caused by root- lesion nematodes, but there was no difference in efficacy compared to fallow in commercial fields under normal agricultural conditions. However, the effects of oriental mustard on nematode populations remained undetermined during both trials. The ineffectiveness of oriental mustard in reducing nematode populations in the summer cultivation trial (experiment 2) was possibly due to the low initial nematode population. In the spring trial (experiment 1), although higher nematode populations were observed in oriental mustard plots than in the fallow plot, the root lesion index decreased and marketable taproots increased compared to fallow. The root-lesion nematode presumably remained below the depth of the soil-sampling layer in the fallow plot. Glucosinolates are known to be very water-soluble [29], and Gimsing and Kirkegaard [30] detected both glucosinolates and isothiocyanates in the soil layer below the level of *Brassica* incorporation. Hence, it is not surprising that these compounds were transported below the level of incorporation.

In the experiment 1, oriental mustard breeding line "17-10" increased the population of root-lesion nematodes during the growing season of the plants. This indicates that oriental mustard is the host for the root- lesion nematode. To avoid injury by root-lesion nematodes, oriental mustard is generally not cultivated before susceptible crops. Instead, suppression using oriental mustard bran has been reported, although it is difficult to apply to fields because of its bulk [31]. When we used oriental mustard breeding lines having different sinigrin content, Japanese radish cultivation after "Y-010" yielded larger marketable taproots than after "17-10". Gimsing and Kirkegaard [30] reported the both glucosinolates and isothiocyanates detected in soil were generally related to the amount of glucosinolates added in the incorporated plant material. Thus, using a mustard variety with high glucosinolate content may help control root-lesion nematode levels.

In the experiment 2, we compared incorporation of oriental mustard to that of white mustard, which is generally used as a landscape plant for summer sowing in Hokkaido. The effect on suppression of soil nematode populations was unclear in this experiment, but damage to greater burdock after oriental mustard incorporation was significantly lower than in the white mustard plot. White mustard mainly produces sinalbin in the shoot [32]. Quinsac et al. [33] confirmed that white mustard is a low activity biofumigant because of the lack of degradation of sinalbin into isothiocyanate. Although the green parts and roots of white mustard do contain glucosinolates that are broken down to an isothiocyanate (benzyl glucosinolate) [33], this is more strongly absorbed than 2-propenyl isothiocyanate [34].

In conclusion, the suppressive effect on root-lesion nematode populations of cultivating and incorporating oriental mustard into soil was less than that of antagonistic plants. However, subsequent crops after oriental mustard incorporation were of better or the same quality as in fallow plots, and had lower damage compared to those after white mustard treatment. Mizukoshi [2] reported that populations of *P. penetrans* after sweet corn and white mustard incorporation were much higher than after other green manures. These results suggest that when cultivating subsequent crops that are susceptible to *P. penetrans*, oriental mustard rather than white mustard should be used as a green manure. It is unlikely that biofumigation alone will provide sufficient levels of nematode control, although this method is also useful to manage other soil-borne diseases and weeds. Matthiessen et al. [35] recognized the importance of increasing disruption of the biofumigant tissues using a tractor-drawn tissue-pulverizing implement and adding excess water to the soil. By devising better incorporation methods for oriental mustard in the field, populations of root-lesion nematodes in soils may be reduced in future.

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