SUPPRESSION OF *MELOIDOGYNE INCOGNITA* BY EXTRACTS AND POWDERED FRUIT OF *GLEDITSIA SINENSIS* (CHINESE HONEYLOCUST)

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ABSTRACT

Wen, Y., D. J. Chitwood, B. T. Vinyard, W. Bai, and S. L. F. Meyer. 2017. Suppression of *Meloidogyne incognita* by extracts and powdered fruit of *Gleditsia sinensis* (Chinese honeylocust). Nematropica 47:155-164.

Although the Chinese honeylocust (*Gleditsia sinensis*) is receiving extensive pharmacological investigation because of its use in traditional Chinese medicine, little work has been undertaken to investigate use of *G. sinensis* products as soil amendments or as sources of nematode-antagonistic phytochemicals. In this study, seed pods (fruit) were dried and ground, and an ethanolic extract was prepared and examined for its effects on egg hatch, movement, and viability of *Meloidogyne incognita* in *in vitro* experiments. In addition, the dried fruit powder and the ethanolic extract were both tested in greenhouse experiments for effects on *M. incognita* populations and on growth of pepper (*Capsicum annuum*), and the dried fruit powder was also tested on water spinach (*Ipomoea aquatica*). In the *in vitro* experiments, concentrations of 1.0 and 10.0 mg/ml ethanolic extract of the fruit powder reduced second-stage juvenile (J2) viability by 96.5% to 98.4%; the higher concentration also suppressed egg hatch by 60.3%. In greenhouse pot tests, *M. incognita* population densities on pepper and water spinach were not suppressed by amending the soil with fruit powder or drenching with fruit powder extract (the latter tested only on pepper), as indicated by enumeration of galls/g root and eggs/g root. Additionally, *G. sinensis* fruit powder and extract exhibited phytotoxicity to pepper, resulting in decreased shoot length and fresh weight and root fresh weight. Shoot and root fresh weights of water spinach were also reduced by amendment of fruit powder into soil. Consequently, although *G. sinensis* produces nematotoxic compounds, neither fruit powder nor fruit powder extract applied to soil demonstrated potential as plant-derived sources for suppressing nematode populations in plant roots. Isolation and identification of the nematode-antagonistic compounds in the fruit of *G. sinensis* would indicate whether these chemicals are potential sources of biologically based nematicides.

Key words: amendment, *Gleditsia*, *Meloidogyne incognita*, phytochemical, phytotoxicity

RESUMEN

Wen, Y., D. J. Chitwood, B. T. Vinyard, W. Bai, and S. L. F. Meyer. 2017. Supresión de *Meloidogyne incognita* por extractos y frutos en polvo de *Gleditsia sinensis* (Chinese honeylocust). Nematropica 47:155-164.

Aunque la Chinese honeylocust (*Gleditsia sinensis*) está siendo investigada farmacológicamente debido a su uso en la medicina tradicional china, poco trabajo se ha hecho para investigar el uso de productos de *G. sinensis* como enmiendas del suelo o como fuentes de fitoquímicos antagonistas de nematodos. En este estudio, las semillas de la vaina (fruta) se secaron y se molieron, con la finalidad de preparar un extracto etanólico y evaluar su efecto sobre la eclosión, la movilidad y la viabilidad de *Meloidogyne incognita* en experimentos *in vitro*. Además, el polvo de los frutos secos y el extracto etanólico se evaluaron en experimentos de invernadero para determinar los efectos en las poblaciones de *M. incognita* y en el crecimiento del pimiento (*Capsicum annuum*), y el polvo de frutos secos también se probó en espinaca de agua (*Ipomoea aquatica*). En los experimentos *in vitro*, las concentraciones de 1.0 y 10.0 mg / ml de extracto etanólico del polvo de fruta redujo la viabilidad de los juveniles en segundo estado (J2) en un 96.5% a 98.4%; la concentración más alta también suprimió la eclosión de los huevos en un 60.3%. En las pruebas de invernadero, las densidades de *M. incognita* en pimiento y en espinaca de agua no disminuyeron al enmendar el suelo con polvo de fruta o empapar con el extracto de fruta en polvo (este último solo evaluado en pimiento), como lo muestra los datos de agallas/g de raíz y huevos/g raíz. Además, el polvo y el extracto de frutas de *G. sinensis* tuvo un efecto fitotóxico al pimiento, dando como resultado una menor longitud de los brotes y peso fresco y peso fresco de la raíz. Los brotes y el peso fresco de raíz de la

espinaca de agua también se redujeron con las enmiendas de polvo de fruta en el suelo. En consecuencia, aunque *G. sinensis* produce compuestos nematotóxicos, ni el polvo de fruta ni el extracto de polvo de fruta aplicados al suelo demostraron su potencial como fuentes derivadas de plantas para suprimir las poblaciones de nematodos en las raíces de las plantas. El aislamiento e identificación de los compuestos antagonistas a nematodos en el fruto de *G. sinensis* indicaría si estos productos químicos son una fuente potencial de nematicidas de base biológica.

Palabras clave: enmienda, fitoquímico, fitotoxicidad, *Gleditsia*, *Meloidogyne incognita*

INTRODUCTION

The development of safe and effective methods to minimize the damage caused by plant-parasitic nematodes is the long-term goal of many nematologists. Because of issues involving the deployment of current broad-spectrum chemical nematicides, the "future of nematode control will depend more on integrated techniques that incorporate cultural practices, genetic resistance, and alternative pesticides" (Zasada *et al.*, 2010a). Many of these components depend upon plant biochemistry, as plants provide excellent sources of structurally diverse chemicals as well as materials suitable for incorporation into soils as amendments for reducing populations of pests and pathogens (Chitwood, 2002; Zasada *et al.*, 2010a; Ntalli and Caboni, 2012). Examples of plant-derived amendments investigated for nematode suppression include Brassicaceae-derived compounds, neem, castor, velvetbean, seaweed extract, and sunn hemp, applied in various forms including cakes, green manures, crop residues, and meals (McSorley, 2011).

Medicinal plants, or biological compounds from such plants, have often been investigated as sources of active ingredients for nematicides. The Chinese honeylocust, *Gleditsia sinensis*, is native to China, and multiple biobased products are produced commercially from *G. sinensis*. Various parts of the tree are used in traditional Asian medicine for many diverse remedies (Lian and Zhang, 2013; Lee *et al.*, 2014). The fruit in particular (large seed pods 15 to 25 cm long) has been used in traditional Chinese medicine as an anthelmintic (Lai *et al.*, 2011). Consequently, studies have been conducted on the chemical constituents of the fruits and/or seeds. Examples of identified compounds include alkaloids, amino acids, carbohydrates, fats, flavonoids, galactomannans, glycosides, phenols, proteins, triterpenoid saponins, other saponins, and tannins (Zhang *et al.*, 1999; Gong *et al.*, 2002; Gao *et al.*, 2008; Oleynikov and Rohin, 2010; Wu *et al.*, 2010; Liu *et al.*, 2016a; Liu *et al.*, 2016b; Zhang *et al.*, 2016). The fruits are particularly rich in saponins, and can have 5% or more (w/w) triterpenoid saponins, which are considered the main active compounds in traditional medicine (Xia *et al.*, 2009; Wang *et al.*, 2016).

Gleditsia sinensis fruit is also used as a traditional insecticide in China (Chen and Tseng, 2014). However, neither this application nor use as an anthelmintic has led to research on activity against phytoparasitic nematodes. The only direct examination of *G. sinensis* for effects on plant-parasitic nematodes is one study demonstrating that an undiluted water extract of the fruit could inhibit the motility of *Meloidogyne javanica* and *Pratylenchus vulnus* but was not lethal (Ferris and Zheng, 1999).

The current study was conducted to determine the activity of *G. sinensis* against *M. incognita*. There were two goals of this research. One was to investigate whether *G. sinensis* fruit powder (made from whole, ground fruit) applied as a soil amendment would be efficacious for suppressing *M. incognita*. The second goal was to determine whether extracts made from fruit powder of *G. sinensis* would exhibit nematotoxicity, indicating that it contains compounds with potential for use as plant-derived nematicides. For these two goals, ethanolic extracts were tested in laboratory assays for bioactivity against *M. incognita*, and greenhouse trials were conducted to determine the effects of fruit powder soil amendments and an ethanolic extract soil drench on plant vigor and on *M. incognita* population densities.

MATERIALS AND METHODS

Nematode cultures

Cultures of *M. incognita* race 1, originally isolated from Salisbury, MD, soil, were used for microwell assays and greenhouse trials. The nematodes were maintained on pepper (*Capsicum annuum*) cv. PA-136 in greenhouse pots. Surface-sterilized eggs and second-stage juveniles (J2) for microwell assays were collected according to Meyer *et al.* (2006). Briefly, egg masses were collected from plant roots and rinsed three times with sterile distilled water. The egg masses were agitated for 3½ min in 0.6% sodium hypochlorite, and then the surface-sterilized eggs were pipetted onto a 500-mesh sieve (25-µm-diam. pore size), rinsed with water, placed

into a sterile vial, refrigerated overnight at 7°C, and used the following day for assays. Additional sterilized eggs were placed on a Spectra/Mesh Nylon Filter (Spectrum Laboratories Inc., Rancho Dominguez, CA) with 30-µm-diam. openings in an autoclaved storage dish, and J2 that passed through the filter within 72 h were collected and used immediately for assays. For U.S. greenhouse tests, eggs of the same *M. incognita* isolate were obtained from ca. 3-mo-old plants, following procedures in Meyer *et al.* (2011), as eggs for greenhouse tests do not need to be surface-sterilized.

For greenhouse trials in China, *M. incognita* (race not determined, but race 1 is the most common in southern China; Liao *et al.*, 2003) was originally isolated from soil from Shenzhen, Guangdong province, and was maintained on tomato (*Solanum lycopersicum*) in greenhouse pots. Egg masses were removed from galls manually, placed in water, and transferred to an incubator (25°C) for 7 d to collect J2 for subsequent use.

Preparation of G. sinensis *fruit powder and ethanolic extract from fruit powder*

Mature fruit (seeds and pods) of *G. sinensis* was collected in Hubei Province, China, air-dried, and ground in an electric mill (model FY 130, Tianjin Taisite, China) to a powder fine enough to pass through a 40-mesh sieve (425-µm-diam. pore size). This fruit powder was either used directly as a soil amendment in greenhouse experiments, or it was extracted with ethanol to determine nematotoxicity of extracted compounds. For extract preparation, 200 g of fruit powder was soaked in 1.6 L of 95% ethanol (12.5% w/v) for 5 days, and then the ethanol extract was filtered through a Whatman® #2 filter paper (Whatman International Ltd, Maidstone, England). The residue in the flask was re-extracted as above with 1.0 L of 95% ethanol. This second extract was then filtered as above and combined with the first extract. The pooled extracts were concentrated in a rotary evaporator (Büchi Rotary Evaporator, Model RE, Brinkmann Instruments, Inc., Westbury, NY). After drying, this resulted in a total of 52.2 g ethanolic extract.

Microwell assays

Microwell assays testing *G. sinensis* extracts for activity against *M. incognita* were conducted in 24-well polystyrene plates, with procedures similar to Wen *et al.* (2013). For microwell experiments, 0.5 g of the *G. sinensis* extract was dissolved in warm deionized water and diluted in 50 ml to obtain a concentration of 10 mg/ml; this solution was passed through a syringe filter (25-mm-diam., 0.2-µm pore size, Whatman) and diluted further as needed for assays. Approximately 100 surface-sterilized *M. incognita* eggs or 50 J2 in 0.1 ml deionized water were placed into each well, and then each well received 0.9 ml of extract or water. Microwell assay treatments were: 1) 10 mg/ml extract solution; 2) 1 mg/ml extract solution; 3) 0.1 mg/ml extract solution; and 4) deionized water control. The pH of each treatment was recorded. A plastic adhesive sheet was placed on each plate and the plates were incubated at 28°C. Each treatment was tested in two trials (three trials at Day 1), with five or ten replicate wells per trial. Second-stage juveniles immersed directly into the treatments were later incubated in a water rinse to determine whether the extracts were nematotoxic or nematostatic. For assays with hatched J2 immersed directly into treatments, numbers of active and inactive J2 were counted after 1 d, 2 d, 3 to 4 d, and 3 to 4 d + 3-d water rinse. Second-stage juveniles that were still active after the water rinse were considered viable. For the assays with immersed eggs, the numbers of J2 that hatched from eggs, and numbers of active and inactive J2, were counted after 3 d. Second-stage juveniles that exhibited body movement were considered active; those that did not were inactive.

Greenhouse trials in the United States

Gleditsia sinensis treatments were either a fruit powder amended into soil, or ethanolic extract from fruit powder dissolved in water and applied as a drench. Since extracts might not contain all of the compounds in fruit powder, both were tested to determine if either would demonstrate activity against nematodes in soil tests. Treatments were: 1) 8 g *G. sinensis* powder/pot (0.8% dry weight/weight dry soil) + *M. incognita*; 2) 12 g *G. sinensis* powder/pot (1.2% w/w) + *M. incognita*; 3) 16 g *G. sinensis* powder/pot (1.6% w/w) + *M. incognita*; 4) a drench of 60 ml water + 60 ml *G. sinensis* ethanolic extract, made by dissolving 24 g extract in warm, deionized water and then diluting to 960 ml with deionized water (6.0% vol/weight soil) + *M. incognita*; 5) control without *G. sinensis* treatment + *M. incognita*; and 6) control without *G. sinensis* treatment or nematodes.

Pepper seeds were planted in starter mix (Premier Pro-mix®, Premier Horticulture Inc., Quakertown, PA). Roots of pepper seedlings (33- to 34-d old) were dipped in water to remove potting mix, and seedlings were then transplanted into 15-cm-diam. pots (1 seedling per pot) that had each received 1 kg of steamed, dried greenhouse soil mixture (16 sand:9 compost). *Gleditsia sinensis* fruit

powder treatments were amended into the soil post-steaming, just prior to seedling transplant. Seedlings received 120 ml water per pot, which was 70% of the water-holding capacity of the soil; the exception was Treatment #4, which received a drench of 60 ml water + 60 ml extract per pot. Soil in each pot was then inoculated with 5,000 *M. incognita* eggs (applied to three holes, each 3-cm deep, made in the soil near the plant roots); water alone was used for treatment #6. There were eight replicate pots per treatment in each of two trials (N = 16), and the pots were arranged in a randomized complete block design.

The greenhouse temperature was maintained at 21°C to 26°C, with natural and supplemental lighting combined for a 15-h day length. Plants were harvested 8 weeks after transplant. Eggs were extracted from roots in 0.6% sodium hypochlorite (Meyer *et al.*, 2011) and counted. Shoot lengths, shoot fresh and dry weights, root fresh weights, and numbers of galls on roots (up to 100 per root system) were determined.

Greenhouse trials in China

Gleditsia sinensis was added to soil as a fruit powder. Treatments were: 1) 0.5% dry weight *G. sinensis* powder/weight dry soil + *M. incognita*; 2) 0.75% w/w + *M. incognita*; 3) 1.0% w/w + *M. incognita*; and 4) control without *G. sinensis* treatment + *M. incognita*.

Water spinach (*Ipomoea aquatica* cv. Taiguokongxingcai) seeds were planted in sand: potting mix 1:4 v/v (potting mix was Liang Tu® (Good Soil), Juyuan Horticulture Ltd. Company, Guangdong Province, China). Three-week-old seedlings were transplanted into 16-cm-diam. pots (1 seedling per pot in Trial 1; 3 seedlings per pot in Trial 2) that had each received 1 L of steamed, dried sandy soil (sand:soil 1:4 v/v). *Gleditsia sinensis* fruit powder treatments were amended into the soil post-steaming, just prior to seedling transplant. One week later, soil in each pot was inoculated with 1,000 J2 per plant, which were added to three holes near the plant roots. There were 10 replicate pots per treatment in each of the two trials (N = 20), and the pots were arranged in a randomized complete block design.

The greenhouse temperature was maintained at 28°C to 34°C during the day and 22°C to 28°C at night under natural lighting. Plants were harvested 45 d after inoculation. To extract eggs, roots were washed in water and then shaken in 1.0% sodium hypochlorite. Eggs were collected on a 500-mesh sieve under running water and counted, and shoot and root fresh weights and numbers of galls on roots were determined. Trial 1 was conducted with one seedling per pot and Trial 2 was conducted with three seedlings per pot, with values calculated per plant.

Statistical analysis

Data were analyzed using SAS v 9.4 (SAS Institute, Cary, NC) PROC GLIMMIX to fit models using the statistical distribution most appropriate for each variable. Most variables were modeled using a negative binomial distribution with log link. Additionally, an offset of log total hatched was specified to obtain estimates for % active, % viable, and total hatched as % of the water control. For the variables, galls/g root fresh weight and eggs/g root fresh weight, log root fresh weight was specified as the offset. Because shoot and root fresh weights were often close to zero, a gamma distribution with log link was used to ensure all weight estimates were non-negative. Shoot length was the only variable that was accurately modeled using the normal distribution. All analyses were conducted both by combining data observed from all trials in a 2-way Treatment × Trial ANOVA and by conducting a separate 1-way ANOVA on data from each individual trial. Comparisons among treatment means, using Tukey-Kramer's multiple comparisons adjustment ($\alpha = 0.05$), were examined for both combined trials and separate trials analyses. Because the biological interpretation was consistent between the combined trials and separate trials analyses, results from the combined trials analyses are reported.

RESULTS

Microwell assays

The pH values of the *G. sinensis* extracts were 6.2 to 7.6 for 0.1 mg/ml extracts, 5.0 to 5.3 for 1 mg/ml extracts, and 3.9 for 10 mg/ml extracts.

Activity of J2 immersed directly into extracts from fruit of *G. sinensis* was affected by treatment and by incubation time. Within 1 d of immersion, J2 activity decreased with increasing extract concentration (Table 1). The lowest J2 activity was in the highest extract concentration (10 mg/ml), with a 31% reduction compared with the water control. After 2 d of incubation, nematode activity decreased in all treatments compared with Day 1. At Day 2, J2 activity in the lowest extract concentration (0.1 mg/ml) was significantly different from activity in water, but was only reduced by 5.7%. However, more than half of the J2 were inactive in the two higher extract concentrations, with activity reduced by 53.7% to 55.7%. After 3 to 4 d of incubation in the extracts, J2 activity was lower than at 2 d and was again

Table 1. Percentage of active and viable *Meloidogyne incognita* second-stage juveniles (J2) immersed in extracts from *Gleditsia sinensis* fruit powder.

Treatment	1 day[x] % active J2	2 days[y] % active J2	3 to 4 days[y] % active J2	3 to 4 days + 3-day water rinse[y] % viable J2
10.0 mg/ml	64.4 dA[z]	37.4 cB	18.4 cC	2.4 cD
1.0 mg/ml	78.4 cA	39.1 cB	12.1 dC	1.1 cD
0.1 mg /ml	88.0 bA	79.6 bB	62.2 bC	53.4 bD
Water	93.6 aA	84.4 aB	72.8 aC	69.3 aD

[x]Data from three trials; N = 20 for combined trials.
[y]Data from two trials; N = 15 for combined trials.
[z]Similar lower case letters indicate that means are not significantly different within a column; similar upper case letters indicate that means are not significantly different within a row. Percentage estimates were obtained from a Treatment × Time ANOVA using a negative binomial distribution with log link and an offset of log total hatched. Pairwise comparisons among the percentage estimates used Tukey-Kramer's multiple comparisons adjustment (α = 0.05).

Table 2. Percentage of *Meloidogyne incognita* egg hatch and second-stage juvenile (J2) activity after 3 days immersion in extracts from *Gleditsia sinensis* fruit powder.

Treatment	% hatch compared with water control	% active J2
10.0 mg/ml	39.7 c[z]	43.3 c
1.0 mg/ml	79.9 b	59.5 b
0.1 mg/ml	86.7 ab	77.6 a
Water	-	84.9 a

Data from two trials; N = 10 for combined trials.
[z]Similar letters indicate that means are not significantly different within a column ($P < 0.05$). Percentage estimates were obtained from a Treatment ANOVA using a negative binomial distribution with log link and an offset of log total hatched. For % hatched compared with water control, the offset used for each treatment was log total hatched in water control. For % active J2, the offset was log total hatched associated with each individual treatment. Pairwise comparisons among the percentage estimates used Tukey-Kramer's multiple comparisons adjustment (α = 0.05).

significantly inhibited by all extract concentrations compared with the water control. The greatest reduction in J2 activity, 83.4%, was in 1.0 mg/ml extract, compared with 74.7% reduction in 10.0 mg/ml. Second-stage juveniles did not recover after the 3-d water rinses, demonstrating that the J2 were dead rather than merely inactive. The 1 and 10 mg/ml treatments were similar to each other in effects on J2 viability; following the water rinse, there were 96.5% to 98.4% fewer viable J2 than in the water control. In all treatments, J2 activity decreased with time. For example, incubation in the water control resulted in death of 26% of the J2 population by the end of the assay.

In assays with *M. incognita* eggs, hatch and J2 activity decreased in the two highest *G. sinensis* extract concentrations, with the greatest suppression in 10 mg/ml (Table 2). Hatch was inhibited by 60.3% (10 mg/ml) and 20.1% (1 mg/ml), compared with the water control (Table 2). Activity of hatched J2 was suppressed by 49% and 30% in 10 mg/ml and 1 mg/ml, respectively. The lowest extract concentration (0.1 mg/ml) did not affect egg hatch or J2 activity.

Greenhouse trials in the United States

Table 3. Effect of *Gleditsia sinensis* fruit powder and fruit powder extract on pepper (*Capsicum annuum*) growth and *Meloidogyne incognita* root galling and nematode population densities.

Treatment[x]	Shoot length (cm)	Shoot fresh weight (g)	Root fresh weight (g)	Galls/root system[y]	Galls/g root fresh weight[y]	Eggs/root system	Eggs/g root fresh weight
1.6% w/w powder	4.3 d[z]	0.4 d	0.3 d	21 c	71 b	3,343 c	12,060 b
1.2% w/w powder	5.5 cd	0.6 cd	0.4 cd	53 b	144 a	10,796 b	26,308 a
0.8% w/w powder	8.3 c	0.9 c	0.6 c	66 ab	132 a	16,138 b	28,249 a
6.0% vol/w extract	18.1 b	5.0 b	2.2 b	98 a	56 b	59,874 a	27,000 a
Water control	30.9 a	11.2 a	6.3 a	100 a	17 c	79,832 a	12,823 b
Water control, no *M. incognita*	28.1 a	10.4 a	6.2 a	NA	NA	NA	NA

Data from two trials; N = 16 for combined trials.

[x]Soil amendments were dry, powdered *G. sinensis* fruit amendment, weight powder/weight dry soil (w/w). The extract was prepared by soaking dried fruit powder in 95% ethanol, removing the ethanol and dissolving the extract in warm water. The extract drench was 60 ml extract/kg dry soil (vol/weight), equivalent to fruit extract from 6.3 g *Gleditsia* fruit powder. Each plant was inoculated with 5,000 *M. incognita* eggs. Plants were harvested eight weeks after inoculation.

[y]Gall numbers were counted up to 100.

[z]Similar letters indicate that means are not significantly different within a column ($P < 0.05$). Growth parameter estimates for each treatment were obtained from a Treatment ANOVA using the distributions: normal for shoot length; gamma with log link for shoot and root fresh weights; negative binomial with log link for galls and eggs per root system; and negative binomial with log link and offset log root fresh weight for galls/g root fresh weight and eggs/g root fresh weight. Pairwise comparisons among the percentage estimates used Tukey-Kramer's multiple comparisons adjustment ($\alpha = 0.05$).

All *G. sinensis* treatments were phytotoxic to pepper seedlings in the greenhouse (Table 3). The 1.6% powder application, which was the highest rate of soil amendment, reduced shoot lengths by 86.1%, shoot fresh weights by 96.4%, and root fresh weights by 95.2%, compared with the *M. incognita*-inoculated water controls. Effects on plant vigor with the 1.2% powder were similar to phytotoxicity caused by the higher amendment rate. The 0.8% powder was only a little less phytotoxic than the 1.6% powder, with reductions of 73.1% (shoot length), 92.0% (shoot fresh weight), and 90.5% (root fresh weight). The fruit powder extract contained ethanol-soluble compounds from the fruit powder and was toxic to *M. incognita* in the lab assays. This treatment reduced shoot lengths by 41.4%, shoot fresh weights by 55.3%, and root fresh weights by 65.1% when applied as a soil drench (Table 3).

Total gall and egg numbers on pepper root systems were generally decreased by *G. sinensis* fruit powder amendments, but this was due to phytotoxicity that reduced root fresh weights (Table 3). The highest numbers of galls and eggs per root system were recorded from the water control plants, and the lowest numbers from the 1.6% w/w *G. sinensis* fruit powder soil amendment. The number of galls per root system was decreased by 79% in the 1.6% w/w treatment, and the number of eggs by 95.8%. However, because root fresh weights were greater in the water control than in the *G. sinensis* treatments, the numbers of galls/g root fresh weight and eggs/g root fresh weight were not suppressed by any *G. sinensis* treatment. Galls/g root in the 0.8% and 1.2% *G. sinensis* soil amendments were 7.8 to 8.5 times higher than in the water control, while eggs/g root doubled in all *G. sinensis* treatments except the 1.6% powder. The 1.6% w/w soil amendment was the only treatment that consistently resulted in low total egg numbers and eggs/g root, although the eggs/g root were not significantly lower than in the water controls.

Greenhouse trials in China

Greenhouse tests conducted with water spinach and *G. sinensis* fruit powder as a soil amendment also demonstrated phytotoxicity, with no suppression of nematode populations on plant roots (Table 4). All three of the tested amendment rates reduced shoot and root fresh weights compared with the water control. The 1% powder decreased shoot fresh weights by 60% and root fresh weights by 54.5%,

Table 4. Effects of *Gleditsia sinensis* fruit powder on water spinach (*Ipomoea aquatica*) growth and *Meloidogyne incognita* root galling and nematode densities.

Treatment[y]	Shoot fresh weight (g)	Root fresh weight (g)	Galls/root system	Galls/g root fresh weight	Eggs/root system	Eggs/g root fresh weight
1.0% powder	1.0 b[z]	1.5 b	43 b	31 a	2,241 ab	1,636 a
0.75% powder	0.7 c	1.3 b	43 b	34 a	1,831 b	1,385 a
0.50% powder	0.7 c	1.3 b	41 b	34 a	2,263 ab	1,744 a
Water control	2.5 a	3.3 a	93 a	31 a	3,687 a	1,259 a

Data from two trials; N = 20 for combined trials.

[y]Soil amendments were dry, powdered *G. sinensis* fruit amendment, weight powder /weight dry soil (w/w). Each plant was inoculated with 1,000 J2. Plants were harvested 45 days after inoculation.

[z]Similar letters indicate that means are not significantly different within a column ($P < 0.05$). Growth parameter estimates for each treatment were obtained from a Treatment ANOVA using the distributions: gamma with log link for shoot and root fresh weights; negative binomial with log link for galls and eggs per root system; and negative binomial with log link and offset log root fresh weight for galls/g root fresh weight and eggs/g root fresh weight. Pairwise comparisons among the percentage estimates used Tukey-Kramer's multiple comparisons adjustment ($\alpha = 0.05$).

while the lower application rates suppressed plant growth by 72% (shoot fresh weight) and 60.6% (root fresh weight).

Total number of galls on water spinach were lower in all three *G. sinensis* treatments than in the water control (53.8% to 55.9% reductions), but numbers of galls/g root fresh weight did not vary among treatments (Table 4). Numbers of eggs/root system were reduced by 50.3% in the 0.75% *G. sinensis* treatment (compared with the water control), but egg densities did not vary among treatments.

DISCUSSION

In our study, ethanolic extracts prepared from *G. sinensis* fruit powder were antagonistic to *M. incognita*, inhibiting hatch, J2 activity, and viability in *in vitro* assays. Failure to restore J2 motility after a water rinse indicates that the *G. sinensis* extract was nematotoxic, rather than nematostatic. The pH values of the extracts were not sufficiently low or high enough to be lethal to *M. incognita* (Meyer *et al.*, 2004), so the toxicity was likely due to the *G. sinensis* plant chemistry. In the soil, however, the extract did not suppress galling or *M. incognita* egg population densities on pepper in the greenhouse. Similarly, the soil amendments prepared from *G. sinensis* fruit powder did not reduce the numbers of galls/g root or eggs/g root on either pepper or water spinach. In fact, on pepper, these numbers were higher in most treatments than in the water control. Additionally, both *G. sinensis* fruit powder and extract were phytotoxic, suppressing pepper root and shoot fresh weights and shoot lengths. Fruit powder soil amendments also decreased water spinach root and shoot fresh weights.

Our results appear to contradict those reported from a previous study in which *M. javanica* J2 and *Pratylenchus vulnus* mixed life stages were exposed to *G. sinensis* fruit aqueous extracts (Ferris and Zheng, 1999). In that investigation, the nematodes were immersed in extracts prepared from 1 g dried plant material/10 g distilled water, activity was recorded at 1, 3, 5, 8, and 24 h, and the nematodes were then placed in distilled water for another 24 h. In the *G. sinensis* extract, *M. javanica* J2 activity decreased during the 0 to 3 h time, and then increased up to 24 h, fully recovering in the water rinse. *Pratylenchus vulnus* lost activity by 24-h exposure to the extract, but also regained activity in the water rinse. Our study did not examine nematode reactions prior to 24 h, but we found that *M. incognita* J2 were less active in extracts than in water after 24-h incubation, and J2 activity further decreased with each day of incubation. Also, the *M. incognita* J2 were killed by the extracts, as indicated by failure to recover in a water rinse. There are numerous potential reasons for the dissimilarity in results between the two studies, including nematode taxa used for each investigation, source of *G. sinensis* fruit, variations in extract preparation, use of ethanol extracts vs. aqueous extracts, and length of exposure to the extracts.

Although *G. sinensis* extract was nematotoxic to *M. incognita* in our laboratory assays, it was not effective for suppressing nematode populations on plants when applied as a drench in the soil. Fruit powder, which contains compounds that would not be present in such extracts, also did not decrease *M. incognita* population densities. While some of the fruit powder amendments reduced numbers of galls

and/or eggs on the total root system, this was because these treatments were phytotoxic and resulted in low root fresh weights. The nematicidal and phytotoxic activity of the fruit components are likely affected by many factors, including concentration of the drench or amendment, rapid breakdown in the soil, soil type, and interactions with soil microbes.

It is not known which of the compounds produced by *G. sinensis* are antagonistic to *M. incognita*. The activity is possibly due to a mixture of chemical constituents. For example, the fruit contains alkaloids, saponins, and flavonoids, which can all act as nematode toxins (Chitwood, 2002; Gong *et al.*, 2002; Wen *et al.*, 2013). Considering that *G. sinensis* fruit contains a high level of saponins, including at least 19 different triterpenoid saponins (Lian and Zhang, 2013), it is likely that at least some of the bioactivity is due to these compounds. Saponins have been widely studied for nematicidal properties, are antagonistic to *M. incognita* and other nematodes, and are active components of some commercial nematicides (Chitwood, 2002; Zasada *et al.*, 2010b; Giannakou, 2011; Ntalli and Caboni, 2012; Chaieb, 2013). Also, various triterpenoid saponins from *Gleditsia* are known to exhibit cytotoxic and anti-angiogenic effects (Lu *et al.*, 2014; Melek *et al.*, 2014).

Although the nematotoxicity of isolated compounds from *G. sinensis* is unknown, chemical constituents from other members of the legume family (Fabaceae) have been examined for bioactivity. Compounds active against mammalian parasites include a sesquiterpenoid lactone from seeds of *Butea frondosa*, which inhibited glucose uptake in adult *Ascaridia galli*, a triterpenoid from *Glycyrrhiza glabra*, which inhibited motility of *Brugia malayi*, and two triterpenoid glycosides from funicles of *Acacia auriculiformis*, which inhibited motility of *Setaria cervi* (Ghosh *et al.*, 1993; Kumar *et al.*, 1995; Kalani *et al.*, 2013). In studies on Fabaceae-derived saponins and their effects on plant-parasitic nematodes, extracts from *Medicago sativa* that contained saponin mixtures were active against three species of plant-parasitic nematodes: *M. incognita*, *Globodera rostochiensis*, and *Xiphinema index* (D'Addabbo *et al.*, 2011). Mortality varied with incubation time, concentration and nematode taxon. The fruit wall of *Acacia concinna* contains two triterpenoid saponins, sonunin III and sonuside, that inhibited motility of *M. incognita* J2 when the juveniles were immersed in concentrations ranging from 250 µg/ml to 1,000 µg/ml (Meher *et al.*, 1988). Sonunin III, with an LD_{50} of 286 µg/ml, exhibited greater nematotoxicity than sonuside (only 2.5% to 4.2% of the J2 were immobilized). Roots or aerial parts of some species in the Fabaceae contain alcohols, alkaloids or triterpenoid glycosides (i.e., saponins) active against *M. incognita* and the pinewood nematode, *Bursaphelenchus xylophilus* (Chitwood, 2002; D'Addabbo *et al.*, 2011). It should also be noted that along with nematotoxicity, saponins can be phytotoxic (Calle *et al.*, 2016; Stavropoulou *et al.*, 2017), which might account for decreased shoot and root vigor in our greenhouse experiments. However, conclusions about chemicals that are primarily responsible for antinematodal or phytotoxic activity of *G. sinensis* fruit require testing of isolated compounds.

Identification of *G. sinensis* fruit constituents responsible for nematode antagonism would indicate whether these chemicals are potential sources of biologically based nematicides. Because there is also phytotoxicity with these treatments, extensive studies with any identified compounds would determine whether they can be applied in active rates in the soil. This might be approached by soil amendment a week or two prior to planting or transplanting to kill weeds and plant-parasitic nematodes, or by use of a slow-release granule formulation like that used for tea saponins in studies on management of *M. incognita* on cucumbers and tomatoes and *Heterodera avenae* on wheat in the field (Li *et al.*, 2016; Wen, unpublished). Further research is needed to determine whether the *Gleditsia*-derived products can be utilized in a similar manner.

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