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Chia: Host Status for *Meloidogyne incognita* and Activity of Plant Extracts

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Abstract

Chia (*Salvia hispanica* L.) seeds are used for food, drinks, oil, and animal feed, and all plant parts are employed in traditional medicine. The growing demand for the seed has created a need for improved disease management. Plant-parasitic nematodes have been found on other *Salvia* spp., but none have been reported from *S. hispanica*. Chia has also not been tested for production of compounds active against these nematodes. Therefore, aqueous extracts from shoots and roots of six chia lines, Brad's Organic, Cono, E2, G3, G5, and W13.1, were tested in laboratory assays. Some concentrations of all extracts were nematotoxic, killing about one-third of *Meloidogyne incognita* (Kofoid & White) Chitwood second-stage juveniles (J2s) in shoot extracts and up to nearly half of J2s in root extracts. Hatch was generally not affected by the extracts. In greenhouse trials, all six chia lines were hosts of *M. incognita*. Chia line G3 had approximately two times or more eggs per gram of root than Brad's Organic or Cono. When cucumber seedlings were transplanted into soil amended with chopped chia shoots (2.3 or 2.5% weight of fresh shoots/weight of dry soil), galling and egg production on cucumber roots were not suppressed. To our knowledge, this is the first report that chia is a host to *M. incognita* (or any phytoparasitic nematode) and that chia shoots and roots produce compounds active against a nematode.

Keywords: chia, *Meloidogyne*, nematode host, plant extract, *Salvia hispanica*, soil amendment

Chia (*Salvia hispanica* L.) is a crop plant native to Mexico (Valdivia-López and Tecante 2015). Historically, the seeds have been used for food, drinks, oil, and animal feed, and the stems, leaves, roots, and seeds have all been employed in traditional medicine (Bochicchio et al. 2015; Cahill 2003; Valdivia-López and Tecante 2015). Chia seeds are currently popular as a functional food containing antioxidants, fiber, minerals, proteins, vitamins, and oils with a high content of polyunsaturated fatty acids, and the mucopolysaccharide from chia is used as a thickening agent (Amato et al. 2015; Bochicchio et al. 2015; Segura-Campos et al. 2014). These characteristics have led to increased marketing of these seeds by the health food industry and to greater commercialization of chia in South America and Australia. There has also been breeding for lines that can be grown as long-day flowering plants in the United States, the Mediterranean basin, and other regions with temperate climates, including Quebec and Prince Edward Island in Canada (Bochicchio et al. 2015; Kaiser and Ernst 2016; Peiretti and Gai 2009). Generally, chia requires similar conditions for successful growth as soybean (Jamboonsri et al. 2012). It is frost sensitive and flood intolerant. Proper timing of seeding can be used so frost terminates the crop and aids in desiccation, but early frosts can affect seed yield and quality negatively (Ayerza 1995).

The growing demand for the seed and consequent escalation of chia cultivation have created an increased need for management of pests and pathogens on this plant (Bochicchio et al. 2015). We therefore investigated reports of chia infection by phytopathogenic nematodes. Various plant-parasitic nematodes, including *Aphelenchoides* spp., *Hemicycliophora* sp., *Heterodera* spp., *Rotylenchus breviglans*, *Xiphinema denoudeni* (USDA Nematode Collection), *Meloidogyne incognita* (Kofoid & White) Chitwood, *M. arenaria* (Walker and Melin 1998), *M. hapla* (Lisetskaya 1971), *M. javanica* (Jaimand 2013), and *Meloidogyne* spp. (Siddiqui et al. 1973), have been collected from other *Salvia* spp., but *S. hispanica* was not listed as a host. Also, in small unrepeated greenhouse trials, no females or cysts of *Heterodera glycines* Ichinohe (soybean cyst nematode) were found on chia lines Brad's Organic (Brad's) or G3, and in a separate test, only one immature female and a small cyst were on a commercially available chia (TruRoots) (Meyer et al. unpublished). The trials indicated that the tested chias are not hosts for *H. glycines*, but it is not known if the lack of parasitic nematodes reported from chia is due to resistance to many nematode species or because little attention has been paid to this aspect of chia cultivation. As chia acreage expands, this information is important for growers, particularly if the species is susceptible to highly destructive nematodes such as *Meloidogyne* spp.

Chia is also of potential interest in nematode management because some plant-derived compounds are sources of nematicides. Although compounds isolated from chia leaves have not been tested against plant-parasitic nematodes, the shoots, roots, and flowers of other *Salvia* spp. are known to produce essential oils and other components that act against various organisms, including bacteria, fungi, insects, *Leishmania* spp., and yeasts (Ali et al. 2015; Bakkali et al. 2008; Bochicchio et al. 2015; Jassbi et al. 2016; Sepahvand et al. 2015). In regard to nematodes, methanol extracts from *Salvia miltiorrhiza* (red sage) root and essential oils from *Salvia officinalis* (sage) aerial plant parts collected during flowering were either weakly or not nematicidal to *Bursaphelenchus xylophilus* (Barbosa et al. 2010; Choi et al. 2008). Essential oil from stalks and leaves of *S. officinalis* was also not active against *M. incognita* (Ntalli et al. 2010). However,

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soil amendment with ground shoots of *S. officinalis* reduced galling caused by *M. javanica* on tomato (Klein et al. 2012).

Chemical components of chia leaves have been identified, including 42 compounds from leaf oil (Ahmed et al. 1994) and 34 from methanolic leaf extracts (Amato et al. 2015). In addition, fatty acid content has been studied in leaves (Ouzounidou et al. 2015) and herbage samples (Peiretti and Gai 2009). The leaves contain compounds that have been isolated from other plants and have demonstrated nematotoxicity or nematode-repellant activity (Mondal et al. 2015; Ndjonka et al. 2013; Ntalli and Caboni 2012; Ohri and Pannu 2010).

Chia has been collected from several countries but has not been widely used in plant breeding research programs. However, a breeding program established at the University of Kentucky is creating chia lines that can be grown in higher latitudes (long-day plants) (Bochicchio et al. 2015). This program served as the source for testing multiple chia lines for host status to the root-knot nematode (RKN) *M. incognita*, which is a pathogen on many plant hosts and is one of the most economically important plant-parasitic nematodes (Jones et al. 2013). The chia lines were selected for variations in country of origin (where the sample was grown), photoperiod for flowering (long versus short day), seed color, and means of line creation (landrace varieties, natural breeding, mutagen treated, or gamma irradiated to create mutations) to see if there were differences in interactions with *M. incognita* (Table 1). The goals of our research were to investigate, for *M. incognita*: (i) infection and reproduction on *S. hispanica* roots; (ii) differences in host status among chia lines; (iii) effects of chia shoot and root extracts on egg hatch and J2 activity; and (iv) if amendment of chia shoots into soil would suppress nematode populations on a following crop.

Materials and Methods

***M. incognita* inoculum.** Nematode eggs and J2s for microwell assays and greenhouse experiments were collected and prepared as described in Meyer et al. (2016). To summarize, *M. incognita* race 1 was grown on susceptible pepper (*Capsicum annuum* L.) 'PA-136' in a greenhouse, egg masses were removed from 2- to 3-month-old plants, and eggs were separated in 0.6% sodium hypochlorite for 5 min and rinsed with sterile distilled water (SDW). J2s for direct immersion into extracts were collected by placing the sterilized eggs into a hatching chamber (Spectra/Mesh Nylon Filter, openings 25 μm in diameter; Spectrum Laboratories, Rancho Dominguez, CA) in an autoclaved dish on a rotary shaker at 35 rpm for 3 days. Pepper PA-136 was also used as a control in the greenhouse tests with chia to indicate that *M. incognita* was reproducing on a susceptible plant.

Preparation of chia root and shoot extracts. Six chia lines (Brad's, Cono, E2, G3, G5, and W13.1) were grown in a greenhouse at 24 to 29°C with natural and supplemental lighting combined for a 16-h daylength, in air-dried loamy sand-enriched soil (2 parts sand to 1 part soil, v/v; 86.0% sand, 7.4% silt, 6.6% clay). Plants were harvested 1.5 months after planting, as flower buds had begun to form. Shoots and roots were collected, weighed, and stored at –80°C. The plant tissue was then freeze dried (FreeZone 4.5 freeze dryer, Labconco, Kansas City, MO) in small batches and finely ground in a Cyclone Sample Mill (UDY, Fort Collins, CO) equipped with a 1-mm-diameter pore sieve. Procedures for preparing water-soluble extracts were similar to those in Jindapunnapat et al. (2018). For roots and shoots from each of the six chia lines, 3 g of freeze-dried powder was suspended in 30 ml of SDW and placed on a mechanical rotary shaker (VWR, Advanced Digital Shaker, Radnor, PA) at 150 rpm for 24 h at room temperature (25°C). Extracts were then vacuum filtered through Whatman number 1 filter paper (Whatman, Clifton, NJ), dried in a vacuum centrifuge (CentriVap Concentrator, Labconco) at 40°C for approximately 12 h, and resuspended in SDW (0.02 g of dried extract/ml). The solutions were filtered through syringe filters: 1.0 μm, 0.45 μm (Nalgene, Rochester, NY), and 0.2 μm (Whatman). Concentrated extracts were stored at 4°C until use.

Microwell assays of aqueous chia root and shoot extracts for activity against *M. incognita* eggs and J2s. Assays were conducted in 96-well polystyrene plates, following procedures in Meyer et al. (2006). For previously hatched J2 assays, each well received approximately 35 J2s in 10 μl of SDW, followed by 190 μl of extract (200 μl total per well). For assays with immersed eggs, each well received an aqueous suspension of eggs at various developmental stages, including 35 eggs that each contained either a first-stage juvenile (J1) or J2 in 10 μl of SDW. This was followed by 190 μl of extract (200 μl total per well). Each extract treatment (except J2s in shoot extracts) also received 50 μg/ml of kanamycin monosulfate (PhytoTech Lab, Shawnee Mission, KS) to control microbial contamination. This was diluted to 47.5 μg/ml after addition of extracts to nematode suspensions in the wells. The microwell plates were covered by plastic adhesive sealing film (Excel Scientific, Victorville CA), the lids sealed with Parafilm (Bemis, Neenah, WI), and the nematodes incubated at 26°C. Aqueous (SDW) chia extract treatments in each assay were root or shoot extracts from the six chia lines, each at four concentrations: 0.01 g/ml (100%), 0.0075 g/ml (75%), 0.005 g/ml (50%), and 0.0025 g/ml (25%). After adding to the 10 μl of nematode suspension in each well, the final concentrations in the wells were 9.5, 7.1, 4.9, and 2.4 mg/ml. Control treatments were water and water + kanamycin (the latter referred to as water+k; not used for J2 shoot extract assays). Each treatment was placed in four or five replicate wells in each of two trials (eight or 10 wells total). For assays with immersed J2s, counts were made of active J2s (showing any movement within 5 s) and inactive J2s (no movement after 5 s) on days 1 and 2 (and day 3 in the shoot extract assay). Following the day 2 or day 3 count, the J2s were rinsed twice with SDW, incubated in the second SDW rinse, and active versus inactive J2s counted the next day (designated as day 3 rinsed or day 4 rinsed). J2s inactive after the water rinse were considered dead. For immersed egg assays, counts of total hatched J2s and of active/inactive J2s were made on days 2, 5, and 7.

Greenhouse trials with *M. incognita* and chia. Chia seeds from each of the six lines were moistened with water for 20 min and then planted into 10.2-cm-diameter pots containing steamed and air-dried loamy sand-enriched soil (16 parts sand to 9 parts soil, v/v; 85.1% sand, 7.2% silt, 7.6% clay, pH 6.9) at five seeds per pot. The soil was watered and the pots covered with semitransparent plastic to retain moisture. At the same time, Pepper PA-136 seeds were planted in PRO-MIX (Premier Tech Horticulture, Quakertown, PA) to be used as controls, indicating that the nematode was reproducing on a known susceptible plant. After germination, chia seedlings were thinned to one per pot, and 1 month after planting, six chia seedlings per line and four to six pepper seedlings (transplanted into soil) were inoculated with *M. incognita*. Each of these seedlings received 2 ml of an aqueous suspension of approximately 16,000 eggs at various developmental stages that included 5,000 eggs with either a J1 or J2, inoculated into several holes in the soil near the base of each plant. Six chia seedlings per line were left uninoculated. Pots were arranged

Table 1. The six *Salvia hispanica* (chia) lines tested for host status to *Meloidogyne incognita* (root-knot nematode) and effects of shoot and root extracts on *M. incognita* hatch and activity of second-stage juveniles

Line[z]	Characteristics	Place of origin
Short day		
Cono	From landrace Chia Pinta	Argentina
Brad's Organic	From landrace Chia Pinta	Mexico
Long day		
W13.1	Cross of G8 and a white-flowered, white-seeded line from South America	United States
E2	Ethyl methanesulfonate chemical mutagen; from landrace Chia Pinta	United States
G3	Gamma irradiated to create mutations; from landrace Chia Pinta	United States
G5	Gamma irradiated to create mutations; from landrace Chia Pinta	United States

[z] Short day = short day photoperiod for flowering; and long day = long day photoperiod for flowering.

in a randomized complete block design. Chia and pepper plants were harvested 6 weeks after inoculation. Shoot fresh weights, root fresh weights, and the number of galls per root system were recorded. To collect eggs, roots were cut and blended in 0.6% sodium hypochlorite at low speed for 1 min, rinsed with water, and the egg suspension poured through nested sieves (#60/#230; pore sizes 250/63-μm diameter) and collected on a #500 sieve (pore size 25-μm diameter). The eggs were suspended in 40 ml of tap water, diluted, and counted to estimate the number per root system. The experiment was conducted twice.

To determine effects of soil amendment with chia shoots on *M. incognita* populations on a following crop, chia shoots were refrigerated overnight and cut into 1- to 2-cm pieces. Steamed and air-dried loamy sand-enriched soil (as described in the previous paragraph) was amended with 2.3% (trial 1) and 2.5% (trial 2) w/w of the chopped chia shoots by hand mixing and placed into 10.2-cm-diameter pots (five pots per treatment, trial 1; three pots per treatment, trial 2). Soil in five or three additional pots was left without amendment as a control. Each pot then received 5,000 total eggs in 5 ml of water, added to two holes in the soil. One week later, 2-week-old cucumber 'Sweet Slice' seedlings were transplanted from PRO-MIX into the pots at one seedling per pot. Pots were arranged in a randomized complete block design. Cucumber plants were harvested 5 to 6 weeks after planting, and shoot heights, shoot fresh weights, root fresh weights, and egg counts were recorded as described above. Gall indices were recorded using the Daulton and Nusbaum index (1961).

Statistical analyses. For laboratory assays, for each day the variable "percent active" was analyzed as a one-factor generalized linear model from a binomial distribution using PROC GLIMIX (SAS Institute 2018) with the logit link function. The assumptions of the model were checked. The mean comparisons were done with Sidak adjusted *P* values so that the experiment-wise error was 0.05. On day 2 of the assays conducted with eggs immersed in shoot extracts, five chia lines (Cono 75%, E2 25% and 50%, G3 25%, and G5 75%) all had values of 100% active and were not included in the analysis. Confidence limits for these 100% values overlapped the confidence intervals for the non-100% values, showing that there was no significant difference between the non-100% means and the 100% means.

For each day of the assay with eggs immersed in chia root extracts, and days 2 and 7 of the assay with eggs immersed in chia shoot extracts, the variable "hatch" was analyzed as a one-factor linear model using PROC MIXED (SAS Institute 2018). The assumptions of the model were checked. Because there was some variance heterogeneity, the variance grouping technique was used to correct it. For day 5 of the assay with eggs immersed in chia shoot extracts, hatch was better fit as a one-factor generalized linear model from a negative binomial distribution using PROC GLIMIX (SAS Institute 2018) with the log link function.

The treatment means were each compared with either the water+k control (Tables 2 and 3) or the water control (Table 3) using Dunnett's method. Mean comparisons of all the treatments together were done with Sidak adjusted *P* values so that the experiment-wise error was 0.05.

Table 2. *Meloidogyne incognita* egg hatch and second-stage juvenile (J2) activity in aqueous extracts from roots and shoots (stems and leaves) of six chia (*Salvia hispanica*) lines. Eggs were immersed in the extracts.

	Root extracts				Shoot extracts			
Treatment[y]	Day 5[z] hatch	Day 5 % active J2s	Day 7 hatch	Day 7 % active J2s	Day 5[z] hatch	Day 5 % active J2s	Day 7 hatch	Day 7 % active J2s
Water	37.3 ab	97.9 a	49.6 ab	96.2 a	22.1 a	96.6 a	31.3 ac	90.0 ab
Water+k	30.1 ab	96.3 ab	45.2 ab	96.3 a	16.4 ab	95.4 ab	23.5 abde	95.2 a
Brad's 25%	35.4 ab	94.3 abc	48.3 ab	83.4 bcd****	21.9 a	86.9 abcde	33.0 a	72.3 cde****
Brad's 50%	34.0 ab	90.8 abcd	46.6 ab	85.8 bc****	21.5 a	80.8 bcde*	29.6 abd	***65.0 de****
Brad's 75%	35.4 ab	89.8 abcde*	49.6 a	82.1 bcde****	15.9 ab	79.5 bcde*	22.9 abde	70.5 cde****
Brad's 100%	29.6 ab	87.0 bcdef*	35.9 ab	***67.9 fgh****	16.8 ab	79.9 bcde*	23.4 abde	***66.3 cde****
Cono 25%	30.2 ab	96.3 ab	43.7 ab	79.9 bcdefg****	21.4 a	91.8 abc	32.0 ab	80.5 bc***
Cono 50%	33.4 ab	93.0 abcd	49.1 a	83.5 bcd****	20.0 a	86.9 abcde	29.1 abde	77.7 bcde****
Cono 75%	33.6 ab	90.7 abcd	43.7 ab	79.4 bcdefg****	17.1 ab	75.2 de***	23.3 abde	***63.4 e****
Cono 100%	33.4 ab	89.7 abcde	46.3 ab	75.0 cdefgh****	15.4 ab	75.6 cde***	19.8 bcde	***66.5 cde****
E2 25%	32.7 ab	94.6 abc	46.9 ab	82.0 bcde****	16.6 ab	86.5 abcde	25.4 abde	70.9 cde****
E2 50%	40.5 a*	91.4 abcd	52.8 a	82.7 bcd****	15.5 ab	79.8 bcde*	22.1 abde	70.1 cde****
E2 75%	30.5 ab	78.7 ef****	43.8 ab	73.4 defgh****	14.3 ab	79.0 bcde*	19.0 de	***59.4 e****
E2 100%	32.3 ab	77.1 ef****	43.4 ab	***67.4 gh****	15.9 ab	68.5 e****	20.8 abde	***60.8 e****
G3 25%	38.0 ab	96.1 ab	51.8 a	80.7 bcdef****	19.3 ab	89.6 abcd	28.0 abd	80.8 bcd***
G3 50%	39.6 ab	88.2 bcde*	53.3 ab	79.6 bcdefg****	20.3 a	82.1 bcde*	27.3 abde	67.9 cde****
G3 75%	35.1 ab	87.5 bcdef*	49.4 a	75.9 bcdefgh****	17.1 ab	73.7 de***	23.9 abde	70.2 cde****
G3 100%	32.3 ab	76.0 f****	41.5 ab	***64.8 h****	19.4 ab	87.7 abcde	25.9 abde	***66.7 cde****
G5 25%	35.1 ab	92.5 abcd	49.0 a	86.7 b****	20.3 a	92.6 ab	29.8 abd	74.4 cde****
G5 50%	33.9 ab	87.9 bdef*	47.3 ab	76.8 bcdefgh****	18.1 ab	82.1 bcde*	22.5 abde	***65.6 cde****
G5 75%	37.4 ab	92.6 abcd	52.0 a	82.0 bcde****	14.9 ab	84.9 abcde	21.5 bde	***66.9 cde****
G5 100%	30.0 ab	82.9 def***	42.7 ab	75.6 bcdefgh****	11.4 b	83.5 abcde*	17.1 e	***65.0 cde****
W13.1 25%	32.1 ab	93.0 abcd	44.4 ab	74.1 defgh****	21.6 a	87.9 abcd	30.8 ab	71.1 cde****
W13.1 50%	29.3 ab	85.2 cdef***	41.4 ab	75.9 bcdefgh****	16.8 ab	73.9 de***	26.8 abde	***65.9 cde****
W13.1 75%	34.6 ab	85.9 cdef**	47.1 ab	70.8 efgh****	19.4 ab	85.8 abcde	26.8 abde	***65.9 cde****
W13.1 100%	24.7 b	90.5 abcde	33.1 b*	***65.1 h****	18.1 ab	85.5 abcde	26.3 abde	***63.8 e****

[y] After adding the extracts to 10 μl of nematode suspension in each well, the 25, 50, 75, and 100% were 2.4, 4.9, 7.1, and 9.5 mg/ml, respectively. Water+k = water + kanamycin monosulfate. Root extract assays and shoot extract assays were not conducted in the same trials.

[z] For root extracts on days 5 and 7, and shoot extracts on day 7, hatch was analyzed as a one-factor linear model using PROC MIXED. Because there was some variance heterogeneity, the variance grouping technique was used to correct it. For day 5 shoot extracts, hatch was better fit as a one-factor generalized linear model from a negative binomial distribution using PROC GLIMIX with the log link function. For each day, % active J2s in root and shoot extracts was analyzed as a one-factor generalized linear model from a binomial distribution using PROC GLIMIX with the logit link function. Mean comparisons of all the treatments together were done with Sidak adjusted *P* values so that the experiment-wise error was 0.05. The treatment means were also compared with the water+k control using Dunnett's method. Significance levels of treatment means versus water+k control means are indicated by *, **, ***, and **** (denoting $P \leq 0.05$, 0.005, 0.001, and 0.0001, respectively). Means are not comparable among columns. Treatments that resulted in a significant 15 to 29% decrease in % J2 activity compared with the water+k controls are underlined; decreases of 30% or more are in bold italic font.

Data from greenhouse experiments were analyzed with the statistical package JMP 14.2.0 (SAS Institute, Cary, NC). Differences among treatments were determined by ANOVA, and means were compared using the Tukey–Kramer adjustment for multiple comparisons ($P \leq 0.05$). For nonparametric data, a Kruskal–Wallis test and Wilcoxon each pair nonparametric multiple comparisons were used to determine differences among means ($P \leq 0.05$).

Results

Microwell assays of aqueous chia root and shoot extracts for activity against *M. incognita* eggs and J2s. When eggs were immersed in plant extracts, neither hatch nor percent active J2s were affected on day 2 (data not shown). On day 5, hatch was not inhibited by root or shoot extracts, compared with the water+k control (Table 2). Hatch in E2 50% root extracts was significantly higher than in W13.1 100% and was greater than hatch in water+k when compared just with that control. In contrast with the hatch results on day 5, the percentages of active J2s were decreased in some treatments. In root extracts, J2 activity was lowest in E2 75%, E2 100%, and G3 100%, resulting in 18, 20, and 21% significant decreases (respectively) compared with the water+k control. None of the most active chia root treatments resulted in significantly different J2 activity from all other extract treatments. In shoot extracts, J2 activity on day 5 was reduced in several treatments compared with the water+k control. The strongest effects were in Cono 75% and 100%, E2 100%, G3 75%, and W13.1 50%, with 21 to 28% decreases in percent active J2s.

By day 7, there continued to be little significant effect on hatch in root or shoot extracts (Table 2). In root extracts, only W13.1 100% extract inhibited hatch, when compared only to the water+k control (27% decrease). Hatch remained highest in E2 50%. Hatch in shoot extracts was not significantly decreased compared with water+k on day 7. However, compared with the water control on day 7, hatch was inhibited 37 and 45% by E2 75% and G5 100%, respectively. In contrast with hatch, percent J2 activity in root and shoot extracts decreased in more treatments than on previous days. In root and shoot extracts, all treatments resulted in significantly fewer active J2s than the controls, with a trend toward lowest percent active J2s in the highest extract rate of each chia line. The higher concentrations tended to result in J2 activity reductions of 30% or more, compared with the water+k control. Treatments that resulted in 30% or more loss in J2 activity in both root and shoot extracts were Brad's 100%, E2 100%, G3 100%, and W13.1 100%.

When previously hatched J2s were immersed in root extracts, J2 activity decreased in all treatments compared with the water+k control (Table 3). On days 1 and 2, the lowest J2 activity was generally found in the 100% extract concentrations. Significant decreases in J2 activity on day 1 in the 100% extract rates ranged from 31 to 41% (in G5 100% and Cono 100%, respectively) compared with water+k. On day 2, significant decreases in J2 activity in 100% extracts ranged from 30 to 55% (in G3 100% and W13.1 100%, respectively). However, on day 2, only 75% and 100% G3, G5, and W13.1 resulted in significantly lower J2 activity than in the 25% extract rates of the same chia lines. After the water rinse, J2 viability was decreased by 15% or more in all extracts except Cono 100%, W13.1 75%, and W13.1 100%. In a reversal from results on

Table 3. *Meloidogyne incognita* second-stage juvenile (J2) activity and viability in aqueous extracts from roots and shoots (stems and leaves) of six chia (*Salvia hispanica*) lines. Previously hatched J2s were immersed in the extracts.

	Root extracts			Shoot extracts		
Treatment[x]	Day 1 % active J2s[y]	Day 2 % active J2s	Day 3 rinsed % viable J2s	Day 2 % active J2s[y]	Day 3 % active J2s	Day 4 rinsed % viable J2s
Water	90.4 ab	90.0 a	90.2 ab	86.9 a	84.2 a	80.6 a
Water+k	93.9 a	89.7 ab	93.2 a	NA[z]	NA	NA
Brad's 25%	78.0 cdef****	64.3 efghij****	***54.9 hijk****	74.3 ab*	67.1 abcd*	***53.2 c****
Brad's 50%	74.8 cdefg****	***61.6 hij****	***50.8 ijk****	80.1 ab	67.5 abcd*	67.5 abc
Brad's 75%	***59.0 hi****	***61.7 hij****	66.1 efgh****	81.0 ab	69.0 abcd*	66.0 abc
Brad's 100%	***55.4 i****	***54.7 ij****	74.4 cdef****	67.3 b***	***57.2 cd****	71.2 abc
Cono 25%	72.8 cdefgh****	75.8 cdefg****	***55.4 hijk****	77.9 ab	78.7 ab	***51.4 c****
Cono 50%	76.7 cdefg****	75.5 cdef****	***62.0 fghijk****	71.9 ab*	69.6 abcd*	***55.9 bc****
Cono 75%	***65.7 ghi****	70.6 cdefgh****	70.1 cdefg****	73.3 ab*	64.9 bcd**	69.7 abc
Cono 100%	***55.1 i****	***62.2 ghij****	80.1 bcd****	69.4 ab**	***56.2 d****	76.1 ab
E2 25%	84.0 bc***	70.0 cdefgh****	***59.4 ghijk****	78.1 ab	64.1 bcd***	***55.7 bc****
E2 50%	74.7 cdefg****	***63.2 fghij****	***50.4 ijk****	83.6 ab	62.7 bcd ***	68.4 abc
E2 75%	74.4 cdefg****	68.0 defghi****	62.4 fghij****	80.0 ab	66.9 abcd*	74.3 ab
E2 100%	***63.8 ghi****	***57.7 hij****	68.3 defgh****	77.1 ab	63.0 bcd***	66.9 abc
G3 25%	80.5 bcd****	79.5 bcd**	***48.2 k****	68.5 b**	67.4 abcd*	67.4 abc
G3 50%	76.1 cdefg****	75.4 cdefg****	***58.7 ghijk****	79.5 ab	68.8 abcd*	***56.1 bc****
G3 75%	66.1 fghi****	***63.1 fghij****	70.1 cdefg****	83.7 ab	69.9 abcd*	65.4 abc*
G3 100%	***63.8 ghi****	***62.7 fghij****	74.0 cdef****	71.6 ab*	62.0 bcd***	62.9 abc*
G5 25%	79.7 cde****	81.6 abc*	68.1 defgh****	74.6 ab*	71.9 abcd	57.6 bc***
G5 50%	66.4 fghi****	71.4 cdefgh****	***48.3 jk****	75.6 ab	76.6 abc	64.3 abc*
G5 75%	68.6 defghi****	68.7 defgh****	***65.1 efgh****	76.3 ab	72.0 abcd	63.4 abc*
G5 100%	***64.7 ghi****	***59.3 hij****	70.4 cdefg****	66.3 b***	65.8 abcd**	71.2 abc
W13.1 25%	81.1 bcd****	76.7 cde***	***63.1 fghi****	76.3 ab	71.4 abcd	59.3 bc**
W13.1 50%	74.0 cdefg****	66.3 efghij****	76.6 cde****	81.3 ab	67.8 abcd*	***56.3 bc****
W13.1 75%	67.2 efghi****	***52.9 jk****	81.1 bc****	77.2 ab	68.3 abcd*	63.5 abc*
W13.1 100%	***59.7 hi****	***40.1 k****	81.5 bc****	66.4 b***	***55.5 d****	73.0 abc

[x] After adding the extracts to 10 µl of nematode suspension in each well, the 25, 50, 75, and 100% were 2.4, 4.9, 7.1, and 9.5 mg/ml, respectively. Water+k = water + kanamycin monosulfate (root extracts only). A water+k control was not used for shoot extracts. Root extract assays and shoot extract assays were not conducted in the same trials.

[y] For each day, % active J2s was analyzed as a one-factor generalized linear model from a binomial distribution using PROC GLIMIX with the logit link function. Mean comparisons of all the treatments together were done with Sidak adjusted *P* values so that the experiment-wise error was 0.05. The treatment means were also compared with the water+k control (root extracts) or water control (shoot extracts) using Dunnett's method. Significance levels of treatment means versus control means are indicated by *, **, ***, and **** (denoting $P \leq 0.05$, 0.005, 0.001, and 0.0001, respectively). Means are not comparable among columns. Treatments that resulted in a significant 15 to 29% decrease in % J2 activity or viability compared with the water+k controls are underlined; decreases of 30% or more are in bold italic font.

[z] NA = not applicable.

the previous days, percent viable J2s tended to be significantly lower in the 25% and/or 50% extract rates than in the highest rate from each chia line. In the two most effective treatments, G3 25% and G5 50%, percent viable J2s decreased by nearly half (48%), compared with water+k.

Results with previously hatched J2s in shoot extracts were different from results with root extracts (Table 3). There were no significant effects on J2 activity on day 1 (data not shown) in the shoot treatments. By day 2, several treatments significantly decreased J2 activity by 21 to 24%, compared with the water control (Table 3). Three of these were the highest rate: 100% concentrations of Brad's, G5, and W13.1; one treatment was G3 25%. By day 3, the highest concentration of all shoot extracts significantly suppressed J2 activity compared with the water control, as did many of the lower concentrations. The decrease in J2 activity in G5 100% was significant when compared with water alone but not in the overall analysis with all treatments. Compared with water, J2 activity in the 100% shoot extract treatments was decreased by 22 to 34% (G5 100% and W13.1 100%, respectively). However, following the water rinse, the greatest significant decreases in percent viable J2s were in the lowest extract concentrations, compared with the water control. Concentrations of 25%, 50%, or both from all six chia lines significantly reduced J2 viability by 26 to 36%. Conversely, some J2s in Brad's 100%, Cono 100%, E2 75%, G5 100%, and W13.1 100% may have recovered in the water rinse. However, on all days, the treatments that strongly decreased J2 activity or viability when compared with the control were seldom significantly different in activity from the other extract treatments. Following a water rinse, treatments that significantly decreased J2 activity by 30% or more in both root and shoot extracts were Brad's 25%, Cono 25% and 50%, E2 25%, and G3 50%.

Greenhouse trials with *M. incognita* and chia. In the greenhouse study, shoot fresh weights were similar among chia lines, except that shoots from W13.1 +RKN weighed significantly more than shoots from Brad's +RKN in trial 1, and shoots from E2 –RKN weighed more than shoots from G3 +RKN and W13.1 –RKN in trial 2 (Table 4). In both trials, root fresh weights from chia lines Brad's –RKN and Cono –RKN were significantly higher than root weights recorded from most other treatments. Significant differences in root weights were the same with and without the susceptible pepper +RKN in the analysis (pepper root fresh weights were 16.8 g in trial 1 and 9.3 g in trial 2).

Total numbers of galls per plant were not significantly different among all chia lines and pepper (data not shown). Pepper was only included as a susceptible control to indicate that the nematode was infecting and reproducing in the trials. Galls per gram of root were significantly higher on G3 than on Brad's in both trials (Table 4). G3 had three times as many galls per gram of root as Brad's in trial 1, and 2.5 times more in trial 2. Galls per gram of root were also significantly higher (2.0 times) on G3 than on Cono in trial 2. Galls per gram of root on the susceptible pepper were significantly higher than galls per gram of root on the chia lines in trial 1, but the numbers were not significantly different in trial 2 (65.4 and 237.6 for pepper in trials 1 and 2, respectively).

Total numbers of eggs per plant were significantly lower from all chia lines than from pepper in trial 1, but numbers from all plants were similar to pepper in trial 2 (198,933 and 356,000 for pepper in trials 1 and 2, respectively). When chia lines were compared with each other, G5 had significantly larger numbers (2.1 to 2.4 times more eggs per plant) than Brad's, Cono, or E2 in trial 1. In trial 2, there were no significant differences in eggs per plant with or without pepper data in the analysis.

The numbers of eggs per gram of root in trial 1 were significantly lower on all chia lines than on pepper, whereas in trial 2, the number of eggs per gram of root on G3 was not significantly different from the number on the susceptible pepper host (chia data Table 4; 12,044 and 38,184 eggs per gram of root for pepper in trials 1 and 2, respectively). When eggs per gram of root were compared among chia lines in trial 1, numbers were significantly greater from lines E2, G3, and G5 than from Brad's, Cono, and W13.1 (Table 4). Chia lines G3 and G5 had >3.0 times more eggs per gram of root than Brad's and Cono in trial 1. In trial 2, G3 had significantly more eggs per gram of root (2.2 to 2.6 times) than Brad's or Cono. G3 was therefore consistently higher than Brad's and Cono in eggs per gram of root in both trials.

When cucumber was planted into soil that had been amended with chopped chia shoots or left nonamended, and inoculated with *M. incognita*, no significant differences were found among cucumber plants in shoot length, shoot fresh weight, root fresh weight, total eggs per plant, or eggs per gram of root in either trial (data not shown). Although not significantly different, total eggs per cucumber root system varied in trial 1 from 13,440 (G5 amendment) to 26,900 (nonamended soil) and in trial 2 from 56,000 (Cono) to 121,333 (G5). Eggs per gram of root in trial 1 ranged from 1,297 (G5) to 2,461 (nonamended soil) and in trial 2 from 4,331 (Cono) to 9,320 (G5).

Discussion

Aqueous extracts from chia shoots (stems and leaves) and roots contained compounds active against *M. incognita* J2s. At least one root extract from each of the six chia lines killed one-third or more J2s, with nearly half of all J2s dead in G3 25% and G5 50%. All shoot extracts resulted in loss of J2 activity, and one or two extract concentrations from all chia lines except G5 killed at least one-third of J2s. Hatch was generally not affected by the root or shoot chia treatments.

Table 4. Plant vigor, root galling, and egg numbers on six chia (*Salvia hispanica*) lines in greenhouse trials. Plants were inoculated with *Meloidogyne incognita* (root-knot nematode; +RKN) or uninoculated (–RKN).

	Shoot fresh weight (g)[y]		Root fresh weight (g)[y]		Galls/g of root[y]		Total eggs/plant[z]		Eggs/g of root	
Chia line ± RKN	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1[z]	Trial 2[y]
Brad's, +RKN	14.6 b	13.5 ab	39.0 ab	22.4 abc	11.5 b	127.0 b	33,467 b	233,333 a	868 b	10,142 b
Brad's, –RKN	15.7 ab	13.4 ab	43.5 a	30.5 a	–	–	–	–	–	–
Cono, +RKN	20.2 ab	10.0 ab	49.2 a	17.6 bcd	15.2 ab	159.3 b	37,333 b	211,333 a	757 b	11,615 b
Cono, –RKN	18.6 ab	12.8 ab	47.1 a	25.1 ab	–	–	–	–	–	–
E2, +RKN	15.7 ab	12.2 ab	25.6 c	10.6 d	25.8 ab	226.7 ab	36,933 b	212,800 a	1,445 a	19,114 ab
E2, –RKN	18.5 ab	15.3 a	23.8 c	15.1 cd	–	–	–	–	–	–
G3, +RKN	18.3 ab	7.4 b	23.4 c	9.2 d	34.1 a	319.4 a	65,067 ab	241,333 a	2,744 a	26,016 a
G3, –RKN	18.6 ab	11.3 ab	24.3 c	14.2 cd	–	–	–	–	–	–
G5, +RKN	17.9 ab	9.3 ab	29.1 bc	12.4 d	18.2 ab	186.0 ab	79,600 a	265,200 a	2,730 a	21,484 ab
G5, –RKN	16.0 ab	10.2 ab	24.0 c	14.1 cd	–	–	–	–	–	–
W13.1, +RKN	22.4 a	8.5 ab	41.6 ab	12.8 d	14.2 ab	227.4 ab	54,400 ab	248,667 a	1,273 b	19,172 ab
W13.1, –RKN	17.5 ab	7.8 b	43.9 a	14.4 cd	–	–	–	–	–	–

[y] Means within a column followed by the same letter are not significantly different according to Tukey's adjustment for multiple comparisons ($P \leq 0.05$).
[z] Means within a column followed by the same letter are not significantly different according to a Wilcoxon test with each pair nonparametric multiple comparisons ($P \leq 0.05$).

One exception was W13.1 100% root extract, which suppressed hatch 27% compared with water+k. Shoot extract treatments E2 75% and G5 100% decreased hatch compared with the water control but not compared with the water+k control.

At least 34 metabolites have been identified from *S. hispanica* leaves and 52 compounds from leaf oil, along with fatty acids and other constituents (Ahmed et al. 1994; Amato et al. 2015; Ouzounidou et al. 2015; Peiretti and Gai 2009). Some of these constituents are commonly found in plants and can exhibit nematicidal activity against plant-parasitic nematodes. For example, as reviewed in Jindapunnapat et al. (2018), linalool reduced J2 mobility of *Anguina tritici*, *Heterodera cajani*, *M. javanica*, and *Tylenchulus semipenetrans* (Sangwan et al. 1990), although it was not toxic to *Globodera rostochiensis* or *Globodera pallida* J2s (Būda and Čepulytė-Rakauskienė 2011). Effects on egg hatch were not examined in those papers. Linalool also suppressed galling caused by *M. arenaria* on tomato but did not significantly reduce galling caused by *M. incognita* (Walker and Melin 1996). However, *M. incognita* J2 mobility and hatch were both reduced by linalool (Echeverrigaray et al. 2010; Ntalli et al. 2010). Another example is pinene, which was nematotoxic to *M. incognita* J2s but not to *M. javanica* (Al-Banna et al. 2003). By comparison, essential oil from stalks and leaves of *S. officinalis* (sage), which contains some of the same compounds, was not active against *M. incognita* J2s at doses up to 10 µl/ml (Ntalli et al. 2010). Essential oils from *S. officinalis* aerial plant parts collected during flowering and methanol extracts from *S. miltiorrhiza* (red sage) root were either weakly or not nematicidal to *B. xylophilus* (pinewood nematode) in microwell assays (Barbosa et al. 2010; Choi et al. 2008). A concentration of 2 mg/ml of essential oil from fresh, flowering aerial plant parts of *S. officinalis* resulted in only 0.23% mortality (Barbosa et al. 2010). Methanolic extracts from *S. miltiorrhiza* roots, tested at 1,000 µg/ml, caused just 10% mortality (Choi et al. 2008). The highest rate tested from aqueous *S. hispanica* root or shoot extracts in our current study was 9.5 mg/ml, and some of these extract treatments resulted in higher mortality than those reported from *S. officinalis* oil or *S. miltiorrhiza* root extracts. At this time, it is not known which major constituent, or combination of constituents, from chia may be responsible for the nematicidal activity observed in water extracts from chia roots and shoots.

All six chia lines were hosts for *M. incognita*. Total numbers of *M. incognita* eggs per chia plant were similar to the numbers on a susceptible pepper cultivar in one trial but were lower in another trial. However, eggs per gram of root were generally lower on chia than on the susceptible pepper control (except for G3 in one of two trials). Among the chia lines, G3 had more than two times the number of eggs per gram of root as Brad's or Cono. Despite those differences, if chia were to be planted with suppression of *M. incognita* in mind, it is likely that the total eggs produced per plant would result in similar soil populations regardless of chia line.

Although chia shoots produce compounds active against *M. incognita*, cucumbers transplanted into soil 1 week after amendment with 2.3 or 2.5% chia shoots did not differ in total eggs per root system or eggs per gram of root compared with cucumbers planted in nonamended soil. This is a high rate of soil amendment, corresponding to approximately 46 to 50 t/ha, and was still not efficacious for suppressing *M. incognita* on a following crop. In contrast, dried and ground leaves and stems of *S. officinalis* amended into soil at 1.0% w/w suppressed galling on tomato roots attacked by *M. javanica*, compared with galling on tomatoes transplanted into nonamended soil (Klein et al. 2012). However, the *M. javanica* studies were conducted with amended soil placed into woven bags, buried in small field plots, air dried, and later transferred to greenhouse pots prior to nematode inoculation and seedling transplant into the pots. Because *M. javanica* were added to the soil after it was placed into the pots, the effect was attributed to soil suppressiveness.

In the current greenhouse trials with chia, there was also no indication of an allelopathic effect of shoots on the cucumber plants. However, the 1-week wait from chia amendment to cucumber transplant was planned because of a small preliminary experiment in which shoots and roots of chia plants (seed from TruRoots) were cut and amended into soil. Three pepper seedlings transplanted into the amended soil that same day were smaller at harvest 5 weeks later than three pepper plants in nonamended soil; shoots were 21% shorter and roots 63.5% lower in weight in the former (unpublished).

This is the first time that extracts from chia shoots or roots have been tested for activity against a plant-parasitic nematode. To our knowledge, this is also the first report of *S. hispanica* as a host for any plant-parasitic nematode. Although all six chia lines produced compounds active against *M. incognita* J2s, the nematotoxicity did not result in high suppression (80% or more) of J2 activity, nor was egg hatch generally inhibited in laboratory assays. Constituents in the roots and shoots did not result in resistance to this RKN. Surveys for *Meloidogyne* spp. on chia crops may provide information about distribution and potential damaging effects of *M. incognita* on chia.

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