Responses of *Heterodera glycines* and *Meloidogyne incognita* Infective Juveniles to Root Tissues, Root Exudates, and Root Extracts from Three Plant Species

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Abstract

The infective juvenile (J2) stage of endoparasitic plant nematodes uses plant chemical signals, released from roots, to localize and infect hosts. We examined the behaviors of soybean cyst nematode (*Heterodera glycines*) and root-knot nematode (*Meloidogyne incognita*) J2 in the presence of root signals from marigold (*Tagetes patula*), soybean (*Glycine max*), and pepper (*Capsicum annuum*). Signals were obtained from sources commonly used in phytoparasitic nematode chemotaxis studies: root tips, root exudates, and root extracts. Root tips from each plant species attracted *M. incognita* but *H. glycines* was attracted only to soybean. In contrast, root exudates prepared from marigold, pepper, or soybean

seedlings were attractive to *H. glycines* but were repellent to *M. incognita*. Root extracts had the same effect as exudates. Fractionation of exudates by reversed-phase high-performance liquid chromatography (HPLC) (acetonitrile [CH₃CN] and 0.1% trifluoroacetic acid) revealed highly polar and less polar components affecting behaviors. Fractions eluting at 12% CH₃CN from all three plants attracted *H. glycines* and repelled *M. incognita*. None of the less polar HPLC fractions (>15% CH₃CN) affected *H. glycines* but those from *G. max* and *T. patula* repelled *M. incognita*. Differences among exudates and effects of fractionation on behavior are discussed.

The infective juvenile (J2) stage of soil-dwelling plant-parasitic nematodes must negotiate the complex environment of the rhizosphere, where chemical and other signals influence multitrophic behaviors among soil microorganisms and plants (Bais et al. 2006; Rasman et al. 2012). Chemical cues serve as primary signals for nematode guidance, and prominent among these are plant semiochemicals influencing nematode behaviors important to survival, including host location and infection (Jones 2002; Perry 1996, 1997; Perry and Curtis 2013). The J2 stages of root-penetrating endoparasites such as the soybean cyst nematode *Heterodera glycines* and root knot nematode *Meloidogyne incognita* are highly vulnerable to the risk of starvation and other environmental stresses in the time between hatching and plant entry. Consequently, efficient chemotaxis for location of host plants is crucial. Cyst and root knot nematodes make up the most economically damaging groups of plant-parasitic nematodes (Jones et al. 2013), and understanding their responses to plant root signals is an important component in discovering novel controls. With global losses from plant nematode damage persistently approaching the \$100 billion mark annually (Nicol et al. 2011), and with restrictions placed upon the use of toxic nematicides, the exploration of alternative management strategies, including effects of the massive array of phytochemicals as well as details of nematode chemotaxis (Chitwood 2002; Grundler et al. 1991; Ntalli and Caboni 2012; Reynolds et al. 2011; Wuyts et al. 2006; Yang et al. 2016), has increased urgency.

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Examination of the complex behaviors associated with plant signal detection and nematode chemotaxis through experiments designed to simplify interactions and quantify specific behaviors has been productive (Dalzell et al. 2011; Farnier et al. 2012; Masler et al. 2017; Wang et al. 2010; Winter et al. 2002). These approaches frequently involve plant roots or root preparations to provide chemical signals in vitro. We were interested in comparing the effects of various root preparation types and plant sources on the behaviors of *H. glycines* and *M. incognita* J2. This is the first report of direct comparisons of nematode responses to three sources of root chemicals—root tips, root extracts, and root exudates—from multiple plant species. Evidence is presented for significant differences in *H. glycines* and *M. incognita* response behaviors, and in the chemical nature of plant materials assayed.

Materials and Methods

Nematode cultures. Plants were grown in a greenhouse in sand-filled beakers using the constant moisture system of Sardanelli and Kenworthy (1997) at 26°C and a photoperiod of 16 to 14 h of light and 8 to 10 h of darkness. The southern root-knot nematode *M. incognita* (Salisbury, Maryland, race 1) was reared on pepper (*Capsicum annuum* ‘PA 136’) and the soybean cyst nematode (*H. glycines*, NL1-RHp) was reared on soybean (*Glycine max* ‘Kent’). Inoculation rate was approximately 5,000 eggs plant⁻¹, with soybean harvested at 5 to 7 weeks and pepper at 6 to 8 weeks after inoculation. Cysts were collected by gently rubbing roots under a stream of tap water; then, cysts were rinsed thoroughly in autoclaved tap water (ATW) and crushed to release eggs. Eggs were then collected on a 500-mesh sieve, rinsed with ATW, and transferred to hatching chambers. *M. incognita* eggs were collected by picking egg masses from roots using a jeweler’s forceps, then releasing the eggs by gentle shaking in tap water. Eggs were rinsed with ATW and transferred to hatching chambers comprising 6-well assay plates (Falcon B-D, Franklin Lakes, NJ) with each well-fitted with a sieve. Sieves were prepared using Netwell plate (Corning Costar; Corning Inc., Corning, NY) circular inserts, which have bottom surfaces of 500-μm pore polyester mesh. A circle of 30-μm pore nylon mesh (Spectrum Laboratories, Rancho Dominguez, CA) was secured on top of the 500-μm mesh with a polypropylene ring, and the modified sieves were suspended in plate wells containing 2 ml of ATW. Eggs (approximately 100,000 sieve⁻¹) were added by pipette to the surface of the nylon mesh. Newly hatched J2 passed through the nylon mesh into the plate well and were collected daily. J2 were used in bioassays on the day of collection.

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Plant rearing. Plants used as phytochemical sources included the hosts *C. annuum* and *G. max*, plus marigold (*Tagetes patula* 'Golden Guardian'; Park Seed, Greenwood, SC) for its nematocidal and biological control properties (Hooks et al. 2010) and its behavioral effects on *H. glycines* and *M. incognita* (Masler et al. 2017). We examined the host status of *T. patula* by inoculating it with *H. glycines* and *M. incognita* J2. Neither nematode reproduced on *T. patula*.

Marigold, pepper, and soybean seed were germinated in vermiculite-filled beakers in the greenhouse, and seedlings were maintained at 26°C with a photoperiod of 16 to 14 h of light and 8 to 10 h of darkness. Seedlings were harvested at 10 to 14 days (soybean) or 16 to 20 days (pepper and marigold) after germination. This harvesting approach provided root tips of similar size across the plant species. Roots were washed with tap water and rinsed with double-deionized water (DDW).

Collection of root tips. The terminal 5-mm tips from individual roots of *C. annuum* and *T. patula* root systems were collected. For *G. max*, which has a large taproot with lateral roots, the terminal 5 mm from individual lateral roots were collected. Freshly collected root tips were used for bioassay.

Preparation of root exudates. Seedlings were reared and harvested as described above. Root systems were washed with tap water, rinsed with DDW, and then incubated for 20 h (*G. max*) or 1 h (*C. annuum*, *G. max*, and *T. patula*) at room temperature in 80 ml of DDW. To compensate for differences in the sizes of root systems, incubation densities were typically approximately 0.5 plant system ml⁻¹ of DDW for *G. max* and approximately 4 plant systems ml⁻¹ for *C. annuum* and *T. patula*. Aliquots of the collected liquid (exudate) were dried under vacuum and stored at -20°C. Exudate doses are expressed in plant equivalent (plant eq) volume⁻¹. Preliminary tests on exudate dose and nematode response yielded variable quantitative results. Qualitative results—attraction of *H. glycines* or repulsion of *M. incognita*—were consistent. Doses that yielded consistent quantitative results were used in all subsequent tests.

Preparation of root extracts. With marigold and pepper, entire rinsed root systems were cut into pieces and weighed fresh. For soybean, with its larger root system, only the lateral roots were collected and weighed. Root materials were extracted by hand in DDW using 1.5-ml polypropylene tubes (Axygen Inc., Union City, CA) fitted with polypropylene pestles. Extracts were centrifuged (20 min, 20,000 × g, 5°C) and supernatant volumes were measured. Because fresh root tissue yielded liquid during extraction, supernatant volume was greater than the volume of DDW used in extraction. For assays with fresh supernatant, concentrations are expressed as the original weight of fresh root material in total supernatant volume. A portion of fresh supernatant was used immediately in bioassays, with the remainder dried under vacuum and stored at -20°C. Extract doses in bioassays are expressed as milligrams of plant equivalent (mg eq) volume⁻¹.

Evaluation of extracts of *G. max* lateral roots obtained from different root section positions. To examine for potential differences of J2 responses to materials obtained from locations along the length of the lateral root, soybean roots were collected, rinsed, and cut into several different section types using a sharp scalpel. Three terminal sections containing the root tip (5, 20, and 40 mm from tip back toward the main root) and four nontip sections (5 to 10, 10 to 15, 15 to 20, and 20 to 40 mm from the root tip) were prepared. Sections were counted and weighed, and section types pooled. The seven section pools were extracted in DDW and supernatants were collected as above. Supernatants were used fresh or dried and rehydrated as indicated for each experiment. Extract doses in bioassays are expressed as milligrams of tip section (mg eq) volume⁻¹.

Reversed-phase fractionation. For broad fractionations, dried root exudates were rehydrated in 0.1% trifluoroacetic acid (TFA) and loaded (9 to 17 plant eq) onto a C₁₈ reversed-phase (RP) cartridge (SepPak; Millipore, Billerica, MA). Bound material was eluted in five steps with 1 ml each of 0.1% TFA and an acetonitrile (CH₃CN) series of 10, 20, 40, and 80% CH₃CN in 0.1% TFA. All five fractions were dried under vacuum and rehydrated for bioassay as needed. For expanded separations, dried root exudates were rehydrated in 0.1%

TFA and fractionated on an RP high-performance liquid chromatography (HPLC) system comprising an Agilent HP1200 liquid chromatograph (Agilent Technologies, Santa Clara, CA) fitted with an Agilent Eclipse XDB C₁₈ column (150 mm in length by 4.6 mm internal diameter). Mobile phase (A = 0.1% TFA and B = CH₃CN in 0.1% TFA) was run at 0.25 ml min⁻¹, with 5% B from 0 to 5 min and 5 to 85% B from 5 to 45 min. Typical sample input was 7.5 to 15 plant eq. Fractions (500 µl) were collected at 2-min intervals from 0 through 50 min, and dried under vacuum. Dried fractions were rehydrated as needed for bioassay.

Bioassays. A solution of 23% (wt/vol) Pluronic F-127 gel (Sigma-Aldrich, St. Louis) in DDW was prepared according to Wang et al. (2009b) and stored as liquid at 4°C. To prepare nematodes for assay, infective J2 were suspended in DDW and mixed directly into the F-127 solution at 10°C (necessary to maintain F-127 as liquid). This mixture was dispensed into assay plate wells (1 ml) or assay plates (6 ml) such that J2 were homogeneously distributed. The solutions gelled for 10 min at room temperature, after which samples (root tips, sample dispensers) were added. All assays were conducted at 20°C in the dark.

Root tip bioassay. Attraction assays with root tips were according to Wang et al. (2009b). Briefly, 1 ml of 23% F-127 containing 500 *H. glycines* J2 was loaded into wells of a 12-well tissue culture plate (Corning Inc.), followed by the application of 1 *C. annuum*, *G. max*, or *T. patula* root tip per well. All tips were 5 mm and plates were incubated in the dark at 20°C. Nematodes touching each root tip were counted at 2 and 4 h after assay start, because nematodes were quickly attracted to the root tip. In total, 6 to 12 replicates were included in each experiment, and each experiment was repeated at least twice.

Root extract and exudate bioassays. Bioassays were modified from the methods described by (Wang et al. 2009a). F-127 solution (6 ml of 23% gel) containing 1,800 freshly hatched J2 of *H. glycines* or *M. incognita* were poured into 60-mm Petri plates (U.S.A. Scientific, Ocala, FL) at 10°C. Root extracts or root exudates in DDW were mixed with F-127 to prepare sample solutions at a final gel concentration of 17.3%. Control samples were prepared with DDW in F-127. Sample dispensers were modified 250-µl pipette tips (wide-orifice; Mettler-Toledo Rainin, Oakland, CA), cut 2.3 cm from the small end to accommodate 100 µl of sample. Two sample dispensers (replicates) were placed antiparallel and 1 cm apart in the center of the plate dispenser. Plates were incubated at 20°C for 20 h, which allowed accumulation of J2 within the sample dispensers. J2 in dispensers were examined and counted using a Nikon SMZ-1500 dissecting microscope with NIS-Elements imaging software (Nikon Instruments Inc., Melville, NY). Attraction or repulsion were quantified by calculating J2 in extract or exudate sample dispensers (*S*) as a percentage of J2 in DDW control sample dispensers (*C*): $(nJ2_S - nJ2_C)/nJ2_C \times 100$. Two to three plates (four to six replicates) for each treatment were included in each experiment, and experiments were repeated at least twice.

Statistical analysis. JMP 12 software (SAS Institute Inc., Cary, NC) and GraphPad Prism 7 (GraphPad, LaJolla, CA) were used for the statistical analyses and data exploration. Individual means were compared using Student's two-tailed *t* test and group means were compared by one-way analysis of variance (ANOVA) with Tukey's or Dunnett's multiple comparison tests as indicated.

Results

Response of J2 to root tips from three plant species. *M. incognita* and *H. glycines* J2 were differentially attracted to root tips prepared from *C. annuum*, *G. max*, and *T. patula* (Fig. 1). All three plants attracted *M. incognita* J2 into contact with root tips by 2 h after initiation of the in vitro assay, and no differences were observed among these plants in their levels of attraction to *M. incognita* (Fig. 1). In contrast, *H. glycines* J2 were attracted only to *G. max*. Attraction of *M. incognita* J2 to each of the three plant species increased with time. From 2 to 4 h, percent increases were 68, 104, and 135 (*P* < 0.05) for *C. annuum*, *G. max*, and *T. patula*, respectively. No changes in mean number of *H. glycines* J2 attracted to *G. max* were observed

Fig. 1. Attraction of *Heterodera glycines* and *Meloidogyne incognita* infective juveniles (J2) to root tips from three plant species in vitro. Root tips (5 mm) were prepared from freshly harvested roots and transferred to wells of a 12-well assay plate. Each well contained 1 ml of Pluronic gel, approximately 500 J2, and 1 root tip. Plates were incubated at 20°C and J2 in contact with each tip were counted at 2 and 4 h. Data are expressed as the mean number of J2 (5 to 10 replicates) touching root tips. For each nematode species, means within times and across plants were compared by one-way analysis of variance with Tukey's posttest. Means labeled with different letters are significantly different ($P < 0.0001$). For each species, means within plants and between times were compared using Student's *t* test. Means connected by lines are significantly different at the levels indicated (** and *** indicate $P < 0.005$ and 0.0005 , respectively). Plant-time means were compared between species using Student's *t* test. Means labeled with different numbers are significantly different ($P < 0.0001$).

Fig. 2. Attraction of *Heterodera glycines* and *Meloidogyne incognita* infective juveniles (J2) to *Glycine max* lateral root extracts in vitro. Lateral root sections of 0 to 20 mm (includes root tip) and 20 to 40 mm were prepared as described and fresh extracts were used in the Pluronic gel-sample dispenser assay. Each assay plate contained 6 ml of Pluronic gel, approximately 1,800 J2, and two sample dispensers. After 20 h at 20°C, all J2 within the sample dispensers were counted. Means ($n = 4$ to 10) were compared using Student's *t* test. Within nematode species and across treatments, means labeled with different letters are significantly different ($P < 0.001$).

between 2 and 4 h, and *H. glycines* remained unattracted to either *T. patula* or *C. annuum*.

Response of *H. glycines* and *M. incognita* J2 to *G. max* root tip section extracts. Because *G. max* root tips were attractive to both *H. glycines* and *M. incognita*, we initiated further exploration by assessing the effects of *G. max* root content on J2 behavior, and included nontip sections of the lateral root. Fresh extract of 0-to-20-mm sections of lateral roots (1 eq dispenser⁻¹) was highly attractive to *H. glycines* J2, with a 5.5-fold increase in J2 number over controls ($P < 0.0001$; Fig. 2). In contrast, *M. incognita* J2 were 10-fold less attracted than controls ($P < 0.0001$), suggesting a strong repellent effect. Results were the same with extract of sections (20 to 40 mm) without the root tip (Fig. 2). The terminal 0-to-20-mm section of *G. max* lateral roots was examined further by comparing fresh and dried-rehydrated extracts. Attraction of *H. glycines* was the same for fresh (504 ± 53 J2 dispenser⁻¹ at 20 h) and rehydrated (485 ± 36) samples (Student's *t* test, $n = 4$ to 8, $P > 0.05$), suggesting that the attractants are not volatile. Dried-rehydrated extracts of the terminal 0-to-20-mm section and four subsections (0 to 5 [tip section], 5 to 10, 10 to 15, and 15 to 20 mm) were all equally attractive to *H. glycines*. Adjusting for fresh weights, means were 88 ± 13 , 83 ± 12 , 69 ± 6 , 74 ± 11 , and 72 ± 11 J2 dispenser⁻¹ mg eq⁻¹ respectively, for the five sections ($n = 4$, one-way ANOVA, $P > 0.05$). In subsequent experiments, *G. max* root extracts were prepared using the 0-to-20-mm section of lateral roots.

Response of J2 to root tip extracts from three plant species. Extracts of root systems from *C. annuum* and *T. patula* and 20-mm lateral root terminal sections from *G. max* were examined for attraction and

repulsion effects on *H. glycines* and *M. incognita* J2 at two root mg equivalent levels (Fig. 3). All root extracts were attractive to *H. glycines* at both the 1 and 10 mg eq doses ($P < 0.005$; Fig. 3A). Attraction levels increased between 79% (1 mg eq *G. max*) and 242% (10 mg eq *C. annuum*) relative to controls. In contrast, no extract was attractive to *M. incognita* at either dose (Fig. 3B). In fact, all *M. incognita* test means were significantly below control means ($P < 0.005$) by at least 70%, indicating strong repellency. There were some dose effects with each nematode. For *H. glycines*, attraction increased 1.8-fold between 1 and 10 mg eq with *C. annuum* and 2.4-fold with *G. max* ($P < 0.05$). No differences were observed between the *T. patula* doses. In *M. incognita*, apparent repulsion increased from 1 to 10 mg eq by approximately 1.2-fold ($P < 0.05$) with *C. annuum* and *G. max*.

Response of J2 to root exudates from three plant species. We examined the timing of exudate collection to determine whether reduced incubation times, which would more closely reflect root physiology postharvest than collecting for extended times, can be used in sample preparation. *G. max* root exudates collected at 1 and 20 h after root system soaking were tested for attractiveness to *H. glycines* (Table 1). There was no advantage for attracting J2 by incubating roots for 20 h. In fact, more J2 were attracted to the 1-h exudate than

Table 1. Comparison of effects of *Glycine max* root exudate collection time on attraction of *Heterodera glycines*^z

Root equivalent dispenser ⁻¹	Exudate collection	
	1 h	20 h
Control	80.8 ± 5.0	80.8 ± 5.0
0.01	237.8 ± 27.4 a **	178.3 ± 28.7 a *
0.05	446.3 ± 42.5 a ***	335.8 ± 39.5 a ***
0.1	601.5 ± 24.4 a ****	450.8 ± 46.8 b ***

^z *G. max* root exudates were collected at 1 and 20 h and processed as described. Dried and rehydrated samples were tested in the Pluronic gel plate assay for effects on *H. glycines* infective juvenile (J2) behavior. Assay plates were incubated 20 h at 20°C and all J2 within sample dispensers were counted. Data are expressed as mean J2 dispenser⁻¹ ($n = 4$). Means were compared using Student's *t* test. At each dose and between collection times, means followed by different letters are significantly different ($P < 0.05$). Levels of significance of test means versus control mean are indicated (*, **, ***, and **** indicate $P < 0.05$, 0.005, 0.001, and 0.0001, respectively).

Fig. 3. Response of *Heterodera glycines* and *Meloidogyne incognita* infective juveniles (J2) to *Capsicum annuum*, *Glycine max*, and *Tagetes patula* root extracts tested at different doses in vitro. Rehydrated root extract supernatants were prepared in Pluronic gel and loaded into sample dispensers as described. Each assay plate contained 6 ml of Pluronic gel, approximately 1,800 J2, and two sample dispensers. Doses tested were 1 or 10 root milligrams of plant equivalent (mg eq) dispenser⁻¹, as indicated. After 20 h at 20°C, all J2 within dispensers were counted. Data are expressed as the mean number of J2 in extract sample dispensers as a percentage of J2 in control sample dispensers. Means were compared using Student's *t* test ($n = 4$ to 6; * indicates that *M. incognita*-*T. patula* was not tested at 1 root mg eq). For each nematode, means within plants and between doses labeled with different letters are significantly different ($P < 0.005$). Means within doses and between plants that are labeled with different numbers are significantly different ($P < 0.005$). Horizontal dashed lines indicate response level equal to control. Values significantly greater than the control indicate attraction and those significantly less than the control indicate repulsion.

Fig. 4. Attraction of *Heterodera glycines* and *Meloidogyne incognita* infective juveniles (J2) to *Capsicum annuum*, *Glycine max*, and *Tagetes patula* root exudates in vitro. Fresh root systems were rinsed and incubated for 1 h at room temperature in double-deionized water, as described. Exudates were recovered, dried under vacuum, rehydrated in Pluronic gel-water, and loaded into sample dispensers as described. To adjust for differences in root masses, root exudate concentrations (plant equivalent dispenser⁻¹) were 0.01 for *G. max* and 0.1 for *C. annuum* and *T. patula*. Assays were conducted in 60-mm plastic dishes containing approximately 1,800 J2, as described. After 20 h at 20°C, all J2 within dispensers were counted. Data are expressed as the mean number of J2 in dispensers as a percentage of water control. Means were compared using Student's *t* test ($n = 4$ to 6; for *M. incognita*-*C. annuum*, $n = 2$). For each plant, levels of significant differences between nematodes are indicated (* and **** indicate $P < 0.05$ and 0.001, respectively).

the 20-h exudate at 0.1 plant eq ($P < 0.05$). Consequently, we used 1-h exudates (which were within 2 h of harvest) for all subsequent assays. Exudates from *C. annuum*, *G. max*, and *T. patula* were highly attractive to *H. glycines* (Fig. 4). Percent increases in attraction over water controls ($P < 0.05$) were 156 ± 21 , 627 ± 25 , and 303 ± 8 for *C. annuum*, *G. max*, and *T. patula*, respectively. All *M. incognita* responses were negative ($P < 0.05$), representing decreases of between 80 and 90% relative to water controls (data not shown).

Fractionation of root exudates. Stepwise C_{18} fractions of exudates from three plant species had similar but not identical attractive and repellent effects on *H. glycines* and *M. incognita*, respectively (Table 2). *H. glycines* was attracted ($P < 0.005$) to the most polar fraction (aqueous 0.1% TFA) from each of the plant species. In addition, equally strong attraction ($P < 0.005$) was also stimulated by the least polar fraction tested (80% CH_3CN in 0.1% TFA). Some of the intermediate polarity fractions from *C. annuum* and *T. patula* but not *G. max* were significantly ($P < 0.05$) attractive to *H. glycines*. *M. incognita* was strongly repelled ($P < 0.0005$) by each of the five fractions from *C. annuum* and *T. patula* but only the most polar fractions from *G. max* were strongly repellent to *M. incognita*. The 20 and 40% CH_3CN *G. max* fractions were moderately ($P < 0.005$) repellent whereas the 80% CH_3CN fraction had no effect.

The clear behavioral differences of *H. glycines* and *M. incognita* J2 that were observed with whole exudates were retained by fractionated exudates exposed to low pH and organic solvent. These characteristics were examined further using RP-HPLC with gradient separation. The strong attractive (*H. glycines*) and repellent (*M. incognita*) activities detected in the stepwise separations are present in the column fractions (Fig. 5). In fact, attractive and repellent materials are present in the same two polar fractions (numbers 4 and 5), eluting between 9 and 15% CH_3CN , for all three plants. This is consistent with results from the cartridge tests. It is curious that the relatively weaker *H. glycines* attractive effect of the *G. max* TFA cartridge fraction (versus *C. annuum* and *T. patula*; Table 2) is reflected in the column fractions. Fractions 4 and 5 from *C. annuum* and *T. patula* (Fig. 5A and E) are attractive whereas only *G. max* fraction 4 is attractive (Fig. 5C). Quite unexpected was the absence of any attractive activity in fractions eluting above 15% CH_3CN from all three plants. This is in stark contrast with the stepwise results (Table 2). A similar condition was observed with *M. incognita* repellency by *C. annuum* (Fig. 5B), where only the polar fractions (numbers 4 and 5) were repellent. In contrast, *G. max* and *T. patula* contain repellent materials eluting in both polar and less polar fractions (Fig. 5D and F), with fractions 12 and 13 (41 to 47% CH_3CN) showing consistent effects.

Discussion

H. glycines and *M. incognita* infective J2 exhibit varying behaviors when exposed to plant root signals presented in a variety of

forms and derived from different plant species. These different behaviors expose fundamental chemoreceptive differences between *H. glycines* and *M. incognita*. The large array of chemicals present in and released from roots contain both attractants and repellants to plant-parasitic nematodes (Reynolds et al. 2011; Wuyts et al. 2006; Xu et al. 2015), and the chemical signals expressed can vary not only between plant species but also between same-species cultivars (Yang et al. 2016; Zhao et al. 2000). Our observations with whole root tips presumably illustrate differences in both root phytochemicals and perception by *H. glycines* and *M. incognita*. Because the root tips in our assays were cut from root systems, they represent mechanically disrupted, wounded tissue that may be expected to present different chemical cocktails than those released by intact systems. For example, activation of the ethylene pathway through wounding can invoke varied attraction or repulsion responses by *Heterodera* and *Meloidogyne* spp. (Fudali et al. 2013; Hu et al. 2017). Nevertheless, *H. glycines* and *M. incognita*, with relatively narrow and wide host preferences, respectively (Lilley et al. 2005), behaved rather predictably in our assays with regard to attraction.

The aggressive disruption of root tissue through mechanical extraction should produce samples containing secondary metabolite mixtures to which J2 are not normally exposed. Release of alkaloids, phenolics, proteins, terpenoids and many other phytochemicals can be expected (Bacetty et al. 2009; Chitwood 2002; De-la-Peña et al. 2012; Pegard et al. 2005; Reynolds et al. 2011; Wuyts et al. 2006; Yang et al. 2016). In addition, enzymes such as those which hydrolyze glucosinolates into active products (Gimsing and Kirkegaard 2009) may be activated, adding to the complexity. In this chemical context, it is notable that the two nematodes behave completely differently. The repulsion of *M. incognita* J2 is in complete opposition to their attraction to intact root tips, whereas the attraction of *H. glycines* J2 to root extracts is an extension of their root tip behaviors. Without chemical characterization of the extracts, and with bioassay limitations, specific conclusions regarding the molecules involved in the J2 behaviors cannot be drawn. However, because the results are consistent across three plant species and 10-fold different concentrations, they do, in fact, suggest strikingly different chemoreceptive systems in the two nematodes. Root extract slurries, which contained all materials from disrupted roots (Masler et al. 2017), elicited behaviors from *H. glycines* and *M. incognita* similar to those reported here with extract supernatants rather than to root tips. This suggests that root tissue disruption itself causes the changes in behavior relative to root tips.

Manipulations of root tips illustrate complexities of phytochemical source and nematode behavior. *H. glycines* J2 are strongly attracted to the apical root tip of *G. max* seedlings (Hu et al. 2017) and to cut apical root tips (this work). It was somewhat surprising, then, that extracts from different locations along the lateral roots of *G. max* were

Table 2. High- and low-polarity active components in root exudates of three plant species as detected with *Heterodera glycines* and *Meloidogyne incognita* attraction and repellency bioassays^z

Nematode, plant	Control	TFA	Number of nematode J2 per dispenser			
			Cartridge fraction			
			10	20	40	80
<i>H. glycines</i>						
<i>Capsicum annuum</i>	135	477****	215	348**	352**	517****
<i>Glycine max</i>	190	751**	278	224	487	618**
<i>Tagetes patula</i>	117	409****	295****	181*	257***	485****
<i>M. incognita</i>						
<i>C. annuum</i>	82	4****	25****	42****	25****	6****
<i>G. max</i>	188	16***	36***	84**	111*	129
<i>T. patula</i>	82	3****	7****	16***	5****	8****

^z Root exudates were applied to a C_{18} SepPak cartridge system and eluted stepwise with 0.1% trifluoroacetic acid (TFA) and 10, 20, 40, and 80% concentrations of acetonitrile in 0.1% TFA, as described. Dried and rehydrated fractions were tested in the Pluronic gel plate assay for effects on infective juvenile (J2) behavior. Assay doses were 0.25 plant equivalent dispenser⁻¹ for *C. annuum*, 0.1 for *G. max*, and 0.45 for *T. patula*. Assay plates were incubated 20 h at 20°C, and all J2 within sample dispensers were counted. Data are expressed as mean J2 dispenser⁻¹ ($n = 2$ to 4). For each nematode-plant group, test means were compared against controls by one-way analysis of variance with Dunnett's multiple comparison. Levels of significant differences from control are indicated (*, **, ***, and **** indicate $P < 0.05$, 0.005, and 0.0005 and $P = 0.0001$, respectively).

equally attractive to *H. glycines* J2. Because extracts are the result of aggressive and disruptive methods, and root exudates can be regarded as the products of a more passive and realistic approach, an argument can be made that exudates, although still artificial substitutes for intact roots, more closely reflect what nematodes may be exposed to in the rhizosphere compared with extracts. It is notable that, in examining the effects of potato root diffusate (i.e., exudate) on *Globodera rostochiensis* hatch, Rawsthorne and Brodie (1986) found that, although diffusate was produced primarily by cells at the root tip, active diffusate was nevertheless produced along the entire length of the root. It was revealing, then, that J2 behavior in the presence of exudates in our experiments mimicked the responses to extracts rather than to root tips. Zhao et al. (2000) demonstrated that

M. incognita J2 were attracted to root tips from pea (*Pisum sativum*) and alfalfa (*Medicago sativa* 'Thor') but were repelled by root exudates from these same plants. They identified root tip border cells as the source of attractants, because removal of the cells by washing removed the attraction. Because loss of attractant cannot explain repellency as opposed to simple nonattraction, they cite the presence of positive and negative effectors in root exudates that may affect behavior. Such materials freely accessible in an exudate, as in our experiments, must present a different challenge to the nematode than the root tip.

Yang et al. (2016) demonstrated that the responses of *Meloidogyne incognita* to exudates of three cultivars of tomato were correlated with the susceptibility of the cultivar to *M. incognita*. Exudate of a

Fig. 5. Fractionation of attractant and repellent materials from root exudates of *Capsicum annuum*, *Glycine max*, and *Tagetes patula* by reversed-phase (RP) high-performance liquid chromatography. Exudates were prepared as described, dried, rehydrated in 0.1% trifluoroacetic acid, and injected onto a C₁₈ RP column. Fractions were collected, dried, and dissolved in Pluronic gel-water mixtures for bioassay in 60-mm plastic dishes containing approximately 1,800 infective juveniles (J2), as described. Concentrations of plant equivalent dispenser⁻¹ were 0.13 to 0.25. Data are expressed as the mean number of J2 present in sample dispensers after 20 h at 20°C for each of 25 fractions (open bars) plus controls (ctrl; black bars). Fractions with significant attraction or repulsion versus control are indicated by shaded bars. Data are summarized across four independent experiments for each nematode species-plant combination ($n = 6$ to 8 of each fraction). Fraction means are compared with control means using one-way analysis of variance with Dunnett's. Levels of significance from control are indicated (*, **, ***, and **** indicate $P < 0.05$, 0.01, 0.001, and 0.001, respectively). Numbers in parentheses below x-axes indicate gradient position (% CH₃CN range) of active fractions. The six profiles indicate *Heterodera glycines* × A, *C. annuum*; C, *G. max*; and E, *T. patula*; and *Meloidogyne incognita* × B, *C. annuum*; D, *G. max*; and F, *T. patula*.

highly resistant cultivar repelled *M. incognita* in vitro whereas exudate of a susceptible cultivar was highly attractive. Exudate of a third cultivar of moderate resistance had a neutral effect, neither attracting nor repelling. The authors suggest that differences in root exudate composition can be an important contributing factor to resistance. The sensitivity of J2 to positive and negative signals was illustrated by Reynolds et al. (2011) using intact seedlings of tomato (*Solanum lycopersicum*) and rice (*Oryza sativa*) as exudate sources in a chemotaxis assay system designed to measure J2 directional efficiency in host seeking. Although *M. incognita* and *M. graminicola* were attracted to each plant, the J2 proceeded to their preferred host (*M. incognita* to tomato and *M. graminicola* to rice) by the shortest possible path whereas longer paths were taken to the less-preferred host. The authors suggest that the J2 negotiated complex mixtures of repellents and attractants in selecting the best paths to the host. In addition, they hypothesized that nematodes with relatively broad host ranges (e.g., *M. incognita*) depend upon general plant cues, consisting of appropriate blends and concentrations of semiochemicals, for host location. In contrast, they suggest that nematodes with narrower host ranges (e.g., *M. graminicola* and cyst nematodes) are more specific in their responses to chemical signals.

Exudates from *C. annuum*, *G. max*, and *T. patula* contain attractant and repellent compounds that are water soluble, nonvolatile, and stable at low pH. Stepwise chromatographic separations clearly indicate the presence of highly polar compounds and of compounds of decreasing relative polarities, all of which have effects on behavior. These highly polar compounds consistently elicit strong attraction in *H. glycines* and repellency by *M. incognita*, which is consistent with reports by Papademetriou and Bone (1983) and Diez and Dusenbery (1989) with polar materials eluted from C₁₈ substrates. Papademetriou and Bone (1983) reported that *H. glycines* were attracted to a *G. max* fraction whereas Diez and Dusenbery (1989) reported repellency of *M. incognita* by tomato exudate eluent. The least polar (80%) fractions we collected from each of the three plant species retained the *H. glycines* attractive and *M. incognita* repellent properties, except for the *G. max* noneffect on *M. incognita*. More extensive separations of the 80% fractions by RP-HPLC revealed that much polar material remained and that the early eluting column fractions retained the attraction or repulsion properties of the input samples. It is probable that the early eluting fractions comprised complex mixtures of active attractants and repellants. Additional methods such as those used by Devine and Jones (2003) with potato root leachate are needed for further fractionation and characterization.

Results with fractions eluting at higher CH₃CN concentrations are noteworthy. The complete loss of *H. glycines* attraction may indicate quantitative or qualitative deficiencies. For example, distribution of attractant between neighboring fractions may reduce detectable amounts in each fraction below a necessary minimum. Another possibility is that fractionation separates active compounds that usually act synergistically to attract. Preliminary attempts at fraction mixing (data not shown) have had inconsistent results. Nevertheless, the different chemical mixtures between the 80% input and the subsequent column fractions significantly affected *H. glycines* behavior. This may reflect specific semiochemical mixture requirements by *H. glycines*, similar to the concept suggested by Reynolds et al. (2011). In contrast to *H. glycines* attraction, *M. incognita* repulsion by lower polarity fractions from *G. max* and *T. patula* presents more potential for compound identification, especially because the same two fractions from each species are consistently repellent. The results with *C. annuum* might be explained using the same argument given for the absence of *H. glycines* attraction. It is particularly interesting that the *G. max* fractions are repellent given that the 80% source sample was not. In a variant of the argument of loss of attractiveness with *H. glycines*, fractionation may reduce conflicting signals and allow detection of repellent. Detection of repellency in *G. max* and *T. patula* column fractions is consistent with small lipophilic molecules prepared by solid-phase and solvent extractions of *S. lycopersicum* and *O. sativa* exudates that affect *Meloidogyne* spp. mobility, with possible repellent effects (Dutta et al. 2012).

A major advance in the discovery of natural control compounds will come from understanding the behaviors of plant-parasitic nematodes in the rhizosphere. This chemically complex environment for signal exchange (Baetz and Martinoia 2014; Bais et al. 2006; Chaparro et al. 2013) presents both challenge and reward for compound discovery. Bioassay development incorporating comparative approaches, including varied plant and nematode species and selected plant metabolic ages, will contribute to this discovery.

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