

***Plantago lanceolata* and *Plantago rugelii* Extracts are Toxic to *Meloidogyne incognita* but not to Certain Microbes**

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Abstract: Extracts from the plants *Plantago lanceolata* and *P. rugelii* were evaluated for toxicity to the root-knot nematode *Meloidogyne incognita*, the beneficial microbes *Enterobacter cloacae*, *Pseudomonas fluorescens* and *Trichoderma virens*, and the plant-pathogenic fungi *Fusarium oxysporum* f. sp. *gladioli*, *Phytophthora capsici*, *Pythium ultimum*, and *Rhizoctonia solani*. Wild plants were collected, roots were excised from shoots, and the plant parts were dried and ground to a powder. One set of extracts (10% w/v) was prepared in water and another in methanol. Treatments included extract concentrations of 25%, 50%, 75% and 100%, and water controls. *Meloidogyne incognita* egg hatch was recorded after 7-day exposure to the treatments, and second-stage juvenile (J2) activity after 48 hours. All extracts were toxic to eggs and J2, with *P. lanceolata* shoot extract tending to have the most activity against *M. incognita*. Numbers of active J2 remained the same or decreased in a 24-hour water rinse following the 48-hour extract treatment, indicating that the extracts were lethal. When data from water- and methanol-extracted roots and shoots of both plant species were combined for analysis, J2 tended to be more sensitive than eggs to the toxic compounds at lower concentrations, while the higher concentrations (75% and 100%) were equally toxic to both life stages. The effective concentrations causing 50% reduction (EC₅₀) egg hatch and in J2 viability were 44.4% and 43.7%, respectively. No extract was toxic to any of the bacteria or fungi in our assays.

Key words: *Enterobacter cloacae*, *Fusarium oxysporum* f. sp. *gladioli*, *Meloidogyne incognita*, natural product, *Phytophthora capsici*, *Plantago lanceolata*, *Plantago rugelii*, plantain, *Pseudomonas fluorescens*, *Pythium ultimum*, *Rhizoctonia solani*, root-knot nematode, *Trichoderma virens*.

Species of the genus *Plantago* (commonly known as plantain) have been used for medicinal purposes (Blumenthal et al., 2000) and have also been tested for inhibitory activity against viruses, microbes, and insects. For example, *P. intermedia* extracts were inhibitory to *Escherichia coli* (Uzun et al., 2004), extracts of *P. major* were active against certain viruses (Chiang et al., 2002), bacteria and fungi (Samuelsen, 2000), and a *P. lanceolata* line with high levels of iridoid glycosides enhanced resistance to the insect *Spodoptera exigua* and the fungus *Diaporthe adunca* (Biere et al., 2004).

Studies have also examined activity of several *Plantago* spp. against plant-parasitic nematodes. *Plantago major* reduced damage caused by *Xiphinema index* on grapes (Aballay et al., 2005), and extracts were nematocidal to *X. americanum* and *X. index* (Insunza et al., 2001a, 2001b) and controlled *Ditylenchus dipsaci* on garlic (Insunza and Valenzuela, 1995). *Plantago asiatica* extracts had a negative but reversible effect on activity of *Meloidogyne javanica* and were lethal to *Pratylenchus vulnus* (Ferris and Zheng, 1999). *Plantago lanceolata* plants reduced population densities of *Mesocriconema xenoplax*

on peach seedlings, but also suppressed seedling growth (Whittington and Zehr, 1992).

Except for these studies, little is known about effects of naturally produced *Plantago* compounds on plant-parasitic nematodes. We therefore investigated two species of *Plantago*, *P. lanceolata* and *Plantago rugelii*. *Plantago lanceolata* was selected because it is a common plant with a wide geographic range, is used or is being studied as a pasture and forage plant (Skinner, 2005), and is cultivated for medicines and has exhibited some antimicrobial and nematocidal activity (Whittington and Zehr, 1992; Blumenthal et al., 2000; Biere et al., 2004). *Plantago rugelii* is native to North America and has not been tested for effects on nematodes or microbes, so it was selected as a little-studied species to compare with *P. lanceolata*.

This study was conducted to determine whether extracts from *P. lanceolata* and *P. rugelii* were toxic to *Meloidogyne incognita* (a major nematode pest with a wide geographic and host distribution) and to seven plant-associated microbes. The tested microbes were the beneficial bacteria *Enterobacter cloacae* and *Pseudomonas fluorescens*, the beneficial fungus *Trichoderma virens*, and the plant-pathogenic fungi *Fusarium oxysporum* f. sp. *gladioli*, *Phytophthora capsici*, *Pythium ultimum*, and *Rhizoctonia solani*. The specific objectives were to ascertain whether water- or methanol-soluble extracts from roots and shoots of *P. lanceolata* and *P. rugelii* contained compounds toxic to *M. incognita* eggs or J2 or to the selected microbes and to determine the effective concentration values (EC₅₀) of plant extracts resulting in 50% reduction in activity or 50% mortality of the target organisms.

MATERIALS AND METHODS

Preparation of plant extracts: For the first trial of the experiment, wild *P. lanceolata* and *P. rugelii* plants were

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collected on 13 and 15 September 2005, respectively, from grassy areas at the Henry A. Wallace Beltsville Area Research Center, Beltsville, MD. For both species, the plants were dug from the soil and washed, the flowering and fruiting stalks were removed, and roots were excised from shoots. The roots and shoots were dried at 60°C for 8 d and subsequently refrigerated in plastic bags at 7°C. A second set of plant material was collected and similarly processed for the second trial of the experiment approximately 2 wk after the first collection date for each species.

Dried plant parts were coarsely ground with a mortar and pestle or a Cuisinart Mini-Prep Processor (Model DLC-1BK, Cuisinart, East Windsor, NJ) and subsequently ground to a powder in a Cyclone Sample Mill (Model No. 3010-030, UDY Corporation, Fort Collins, CO). Powdered plant material was stored in a refrigerator at 7°C until use.

Soluble compounds were extracted from the powdered plant material for 24 hr at 4°C in a refrigerated incubator shaker at 100 rpm in deionized (DI) water or in methanol (10% dry weight plant material/volume liquid) in 500-ml Erlenmeyer flasks sealed with Parafilm "M" (Pechiney Plastic Packaging, Chicago, IL) and foil. After the extraction, the suspensions were filtered through eight layers of cheesecloth. Methanol was removed with a rotary evaporator (Büchi Rotary Evaporator, Model RE, Brinkman Instruments, Inc., Westbury, NY) and replaced with a volume of DI water equivalent to the methanol volume recorded after filtering through cheesecloth. All extracts were then centrifuged for 10 min at 3,046g, and the supernatants were filtered sequentially through GD/X series syringe filters (Whatman, Clifton, NJ) using a procedure modified from Meyer et al. (2004). Extracts were filtered through 1.0-µm pore size GF/B filters (and 0.45-µm filters if initial filtering was difficult) and then through sterile 0.2-µm PES filters, dilutions of the plant extracts were made with autoclaved DI water (that had also been filtered through sterile 0.2-µm PES filters), and all concentrations of extracts were filtered again through sterile 0.2-µm filters so that contaminating microbes were removed. Extracts were frozen at -15°C until use. The pH values of the water control and of all extracts were measured on sample portions that were then discarded. The pH values were not at levels toxic to nematodes and were not adjusted.

Meloidogyne incognita assays: *Meloidogyne incognita* race 1, originally isolated from a Maryland field, was cultured in the greenhouse on pepper (*Capsicum annuum*) 'PA-136.' Egg masses were picked from plant roots, collected in tap water, and rinsed three times with sterile DI water. Egg masses were broken apart and eggs were surface-sterilized by agitation for 3½ min in 0.5% sodium hypochlorite. The sterilized eggs were collected, refrigerated overnight at 7°C in sterile DI water, and used the next day for assays. J2 were obtained by plac-

ing sterilized eggs on a Spectra/Mesh Nylon Filter (openings 30 µm in diameter; Spectrum Laboratories Inc., Rancho Dominguez, CA) in an autoclaved storage dish. J2 that passed through the filter within 72 hr were collected and used immediately for assays.

Eight extracts, *P. lanceolata* and *P. rugelii* shoots and roots extracted in water and in methanol, were each tested against eggs and J2 at four concentrations: 25%, 50%, 75%, and 100%. The 25%, 50%, and 75% dilutions were prepared from the original undiluted extracts (prior to addition of egg or J2 suspensions), and the control was 0% (sterile DI water). Nematode eggs and J2 were suspended in water and placed into 24-well cell culture plates (Costar, Corning Incorporated, Corning, NY). Each well received 100 µl sterile DI water containing approximately 200 eggs or 50 J2 and 900 µl plant extract or sterile, filtered DI water control. Undiluted extract (900 µl) added to 100 µl egg or J2 suspension was then designated as the 100% concentration. The culture plates were incubated at 28°C, and three determinations were then made: percentage egg hatch, percentage active J2 in extracts, and percentage active J2 after water rinse. Egg hatch was determined 7 d after placement of eggs into treatments by counting the number of hatched J2 in each well. J2 activity was determined 48 hr after placement of J2 into treatments by counting mobile and immobile nematodes. To establish whether the extracts were affecting J2 viability and not just mobility, the treatments were removed after the 48-hr J2 activity counts and immediately replaced with sterile DI water (water rinse), and J2 activity was determined 24 hr later. Two trials were conducted, with five replicate wells per treatment in each trial.

Microbe assays: The seven microbes exposed to *Plantago* extracts were *E. cloacae* 501R3, *F. oxysporum* f. sp. *gladioli* 99-glad-Y2, *P. capsici* 599, *P. fluorescens* PF5, *P. ultimum* Pucz, *R. solani* R-23A, and *T. vires* GL3 (Howell and Stipanovic, 1980; Roberts et al., 1992; Meyer et al., 2001; Roberts et al., 2005). Bacteria were maintained on nutrient agar (NA) and fungi on potato dextrose agar (PDA) (Sigma Chemical Company, St. Louis, MO). All microbial isolates were from the culture collections of the Sustainable Agricultural Systems Laboratory or the Floral and Nursery Plants Research Unit, USDA-ARS, Beltsville, MD.

PDA and NA were both used for assays with bacteria and fungi. Agar plugs (12-mm diam.) from fungal isolates grown on NA and PDA for approximately 8 d were placed in the centers of plates containing the same medium used to produce the plug. Bacteria were grown overnight in nutrient broth and spread over the entire PDA or NA plate surface. Three discs of Whatman Chromatography #3 filter paper (10-mm diam.) were placed around the periphery of each plate. Approximately 25 µl of each plant extract was placed on two filters in each Petri dish. One filter received 25 µl of sterile water or methanol as a control. Plates were in-

cubated at room temperature and rated daily for zones of inhibition of fungal or bacterial growth around the filter disks. There were three replicate plates for NA and for PDA for each treatment (plant extract \times fungal or bacterial combination). The experiment was performed twice.

Statistical analysis: 1) Model toxicity trends. For each of the 24 observed combinations of nematode stages (eggs, J2 in extracts, and J2 rinsed in water), plant species (*P. rugelii* and *P. lanceolata*), plant parts (roots and shoots), and extraction solvents (water and methanol), the relationship between concentration and percentage hatched eggs or percentage active J2 was modeled by fitting a regression model (SAS, Proc NLIN, Cary, NC) to the 50 observed percentage hatched or percentage active values (i.e., five wells for each of two trials at each of the five observed concentrations). In each case, the appropriate regression model was a log-linear model (percentage hatched eggs or percentage active J2 = $a + b \cdot [\% \text{ concentration} - C]$), a simple linear, or a cubic polynomial model. A single, parsimonious non-linear analysis of covariance model was obtained (Fig. 1, models for eggs and J2 in extract treatments) by combining statistically similar parameters from the 24 regression models using SAS Proc NLMIXED (Milliken and Johnson, 2002).

2) Estimated percentage egg hatch or percentage active J2 at observed levels of each extract concentration, estimated EC_{50} values, compared among the 24 treatments. These estimates are influenced by the count of hatched or active juveniles in each well and by the toxicity trend models. To incorporate this influence and obtain accurate estimates, the observed data were re-sampled by bootstrapping procedures (Efron and Tibshirani, 1993) 500 times for each treatment, extract concentration, and trial.

A single "resampling" was defined as the repeated selection of one well from the five observed wells, replacing the selected well before repeating the selection to obtain a "resample" composed of five wells. For each "resampling" of a treatment, extract concentration, and trial: a) percentage egg hatch or percentage active J2 was calculated, b) a regression model (primarily the log-linear model with some linear, cubic, or Gompertz models) was fitted to the percentages obtained for the five extract concentrations, and c) the regression model provided a predicted percentage egg hatch or percentage active J2 at each observed extract concentration and an estimate of the EC_{50} .

The 500 resamplings and regressions provided 500 individual percentage egg hatch or percentage active J2 estimates and EC_{50} estimates for each treatment and

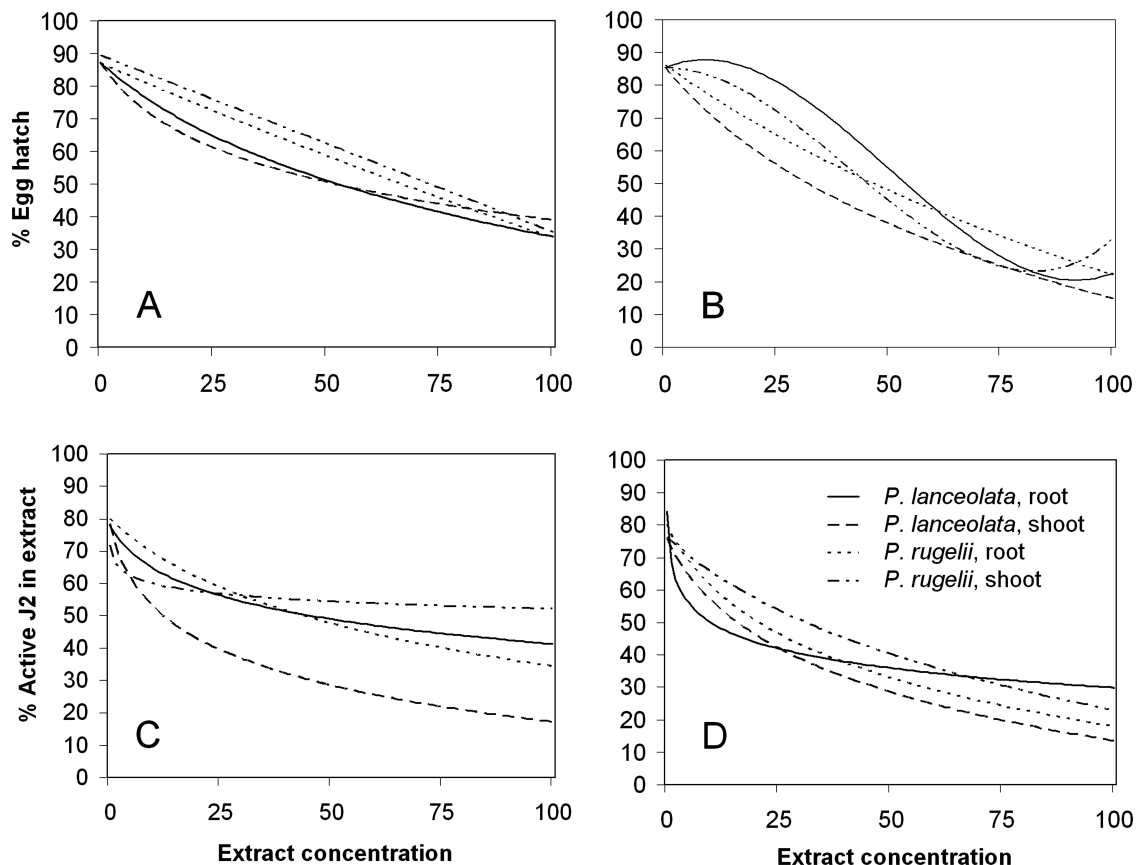


FIG. 1. Toxicity trends showing relationships among *Plantago* extract concentrations and egg hatch or second-stage juvenile (J2) viability of *Meloidogyne incognita*. The single analysis of covariance model described $R^2 = 84\%$ of the total observed data variability with root mean-square-error, $s = 10.28$. Observed data points were 0%, 25%, 50%, 75%, and 100% extract concentrations; the concentration designated as 100% was prepared as 10% w/v and diluted 10% with nematode suspension. Percentage egg hatch determined after 7-d exposure to A) methanol extracts and B) water extracts. Percentage active J2 determined after 48-hr exposure to C) methanol extracts and D) water extracts.

trial. These 500 estimates yielded an extensive representation of how percentage egg hatch or percentage active J2 and EC₅₀ might vary in reality, had 2,500 wells/extract concentration been available for each trial. The average of these 500 estimates, called the bootstrap estimate (Efron and Tibshirani, 1993), was used as the accurate representation for a trial. Hence, for each treatment there were duplicate percentage egg hatches or percentage active J2 estimates at a specific extract concentration and duplicate EC₅₀ estimates that resulted in one value per trial.

The effects of nematode stage, plant species, plant part, and extraction solvent on percentage egg hatch or percentage active J2 were analyzed at specific observed extract concentrations or on the EC₅₀. Four-way ANOVAs with subsequent Sidak-adjusted mean comparisons were conducted, using SAS 9.1.3 Proc MIXED, on the duplicate bootstrap estimates of percentage egg hatch or percentage active J2 or EC₅₀. The GROUP = option of the REPEATED statement was used to define and model heterogeneous within-treatment variances.

RESULTS

Meloidogyne incognita assays: Extracts from *P. lanceolata* and *P. rugelii* roots and shoots decreased *M. incognita* egg hatch and J2 activity. Mean egg hatch and J2 activity were high in the water controls (0% concentration), with values $\geq 79\%$ (Table 1). In the 24-hr water rinse following the 48-hr water control treatment, there was a slight but significant increase to 82.7% active J2. This increase in activity may have been associated with increased aeration during rinsing and indicated that water rinsing did not negatively affect activity of healthy J2. When results from both plant species, from root and shoot extracts, and from both solvents were combined for analysis, the extracts were significantly more toxic to

TABLE 1. Comparison of *Meloidogyne incognita* egg hatch and second-stage juvenile (J2) activity following treatment with extracts from *Plantago lanceolata* and *P. rugelii*, and the effective concentrations causing 50% reduction in egg hatch or 50% J2 mortality (EC₅₀). Data from water- and methanol-extracted roots and shoots of both plant species were combined for analysis.

Nematode stage	Percentage egg hatch and percentage J2 activity ^a					EC ₅₀
	0% extract ^b	25% extract	50% extract	75% extract	100% extract	
Egg ^c	85.1 ab (6.25)	70.2 a (5.52)	49.0 a (3.45)	35.5 a (2.43)	30.3 a (2.66)	44.4 a (5.84)
J2 ^d	79.0 b (1.31)	49.7 b (2.14)	40.1 b (2.49)	33.7 a (2.59)	28.8 a (2.66)	43.7 ab (18.3)

^a Within each column, means followed by the same letter were not significantly different ($P < 0.05$). Letters are not comparable among columns. Means shown were bootstrap estimates from two trials. Because treatments were combined, $n = 16$ for the analysis. Standard errors are in parentheses.

^b All extract concentrations are relative to the 100% concentration (prepared as 10% w/v and diluted 10% with nematode suspension).

^c Percentage egg hatch determined after 7-d exposure to *Plantago* extracts.

^d Percentage active J2 determined after 48-hr exposure to *Plantago* extracts.

J2 than to eggs at the 25% concentration, with less than half of the J2 active after the 48-hr exposure to the extracts (Table 1). Percentage egg hatch and J2 activity both decreased as extract concentrations increased (Table 1), and effects on J2 activity were not reversible in water rinse after any extract treatment (data not shown). This response to the water rinse demonstrated that the extracts were lethal to J2 and were not just rendering them immobile. At the 50% extract concentration, J2 were still somewhat more affected by the treatments than eggs. However, when *M. incognita* eggs and J2 were exposed to 75% and 100% extract concentrations, there were no significant differences in the effects on egg hatch vs. J2 activity, and the EC₅₀ for eggs was similar to the EC₅₀ for J2 (Table 1).

While all extracts were toxic to *M. incognita*, *P. lanceolata* shoot extracts were generally the most toxic to the nematode at all concentrations (Table 2). However, the effects were not significantly different from those recorded with extracts from *P. rugelii* roots. There was an overall difference in effect between the two solvents; extraction with water resulted in greater toxicity to *M. incognita* than methanol extraction, except at the lowest concentration.

With a few exceptions, toxicity trends for individual extracts demonstrated that there was increasing toxicity to eggs and to J2 as extract concentrations increased

TABLE 2. Comparisons of *Plantago lanceolata* and *P. rugelii* root and shoot extracts for effects on *Meloidogyne incognita* egg hatch and second-stage juvenile (J2) activity (data from methanol and water extracts were combined for analysis), and separate comparison of hatch and activity in all treatments extracted in methanol vs. all treatments extracted in water. Percentages of egg hatch, J2 viability in extracts, and J2 viability following water rinse were all combined for analysis and labeled "activity."

<i>Plantago</i> species and plant part, or extraction solvent	Percentage <i>M. incognita</i> activity ^a			
	25% extract ^b	50% extract	75% extract	100% extract
Plant species, plant part				
<i>P. lanceolata</i> , root	52.8 ab (4.3)	42.8 a (2.9)	34.4 ab (2.2)	31.0 ab (2.1)
<i>P. lanceolata</i> , shoot	44.2 b (4.8)	32.4 b (3.4)	24.6 c (2.8)	18.7 c (2.3)
<i>P. rugelii</i> , root	52.4 ab (2.3)	39.3 ab (3.2)	30.8 bc (3.3)	25.6 bc (4.3)
<i>P. rugelii</i> , shoot	61.1 a (4.6)	46.5 a (2.7)	39.6 a (2.6)	37.2 a (2.5)
Solvent				
Methanol	55.7 a (3.0)	45.5 a (2.0)	37.5 a (2.0)	33.5 a (1.8)
Water	49.6 a (2.8)	35.1 b (2.3)	27.3 b (2.0)	22.8 b (2.3)

^a Within each portion of a column, means followed by the same letter were not significantly different ($P < 0.05$). Letters are not comparable among columns nor among "plant species and plant part" vs. "solvent" areas of the table. Means shown are bootstrap estimates for two trials. Because treatments were combined, $n = 12$ for the "plant species and plant part" analysis and $n = 24$ for the "solvent" analysis. Standard errors are in parentheses.

^b All extract concentrations are relative to the 100% concentration (prepared as 10% w/v and diluted 10% with nematode suspension).

(Fig. 1). However, the interactions between the extracts and the nematode did not all follow the same pattern (Fig. 1). When methanol extracts were compared with each other for effects on egg hatch, *P. lanceolata* shoot extract was the most toxic to egg hatch below the 50% concentration, while *P. lanceolata* root extract was the most toxic at the higher concentrations (Fig. 1A). Among the water extracts, *P. lanceolata* shoots were the most active against eggs at all concentrations (Fig. 1B). The water extracts from *P. lanceolata* shoots were more toxic to eggs than the corresponding concentrations of methanol extracts from *P. lanceolata* shoots (Fig. 1A,B). Overall, *P. lanceolata* shoot extract tended to be the most effective treatment for reducing egg hatch at the lowest concentrations, regardless of extraction solvent.

When methanol-extracted treatments were tested for effects on percentage J2 activity, *P. lanceolata* shoot extract was the most toxic to J2 at all observed concentrations (Fig. 1C). In the extracts prepared with water, *P. lanceolata* shoots were the most toxic treatment to J2 at concentrations above 25% (Fig. 1D), although a similar trend in activity against J2 resulted from treatment with water-extracted *P. rugelii* roots (Fig. 1D). Unlike the assays with eggs, *P. lanceolata* shoot extracts exhibited somewhat similar trends in activity against J2 in both solvents (Fig. 1C,D).

The pH of the water controls was approximately 7.10. The pH of the treatments ranged from 4.62 to 5.68, generally increasing a little as each extract was diluted.

Microbe assays: None of the plant extracts at any dilution were inhibitory to *E. cloacae*, *F. oxysporum* f. sp. *gladioli*, *P. capsici*, *P. fluorescens*, *P. ultimum*, *R. solani*, or *T. vires* under the conditions of our filter paper assay, as indicated by the lack of zones of inhibition around any of the filter papers containing the extracts.

DISCUSSION

The eight *Plantago* extracts (*P. lanceolata* and *P. rugelii* roots and shoots extracted in methanol and in water) were toxic to eggs and J2 of *M. incognita*. A water rinse following extract treatment did not result in increased J2 activity, indicating that the extracts were lethal, rather than nematostatic. When all treatments were combined, eggs were not as strongly affected as J2 at lower extract concentrations, but both life stages were equally affected by the 75% and 100% dilutions. However, none of the *Plantago* extracts inhibited growth of the tested bacteria or fungi. Compounds such as the iridoid glycosides aucubin and catalpol, the aglycone aucubigenin, and caffeic acid derivatives including plantamajoside and acteoside have been isolated from *Plantago* spp. and have demonstrated antimicrobial activity (Blumenthal et al., 2000; Samuelsen, 2000). The chemicals extracted in our studies were not identified; they may have been toxic to nematodes and not to the tested microbes, or higher concentrations might be required to visibly affect the plant-associated microbes.

Although the *Plantago* extracts were toxic to *M. incognita*, both *P. lanceolata* and *P. rugelii* are nematode hosts. *Plantago lanceolata* is parasitized by *Aphelenchoides ritzemabosi*, *D. dipsaci*, and *Meloidogyne* sp., and *P. rugelii* by *D. dipsaci* (Buhner et al., 1933; Buhner, 1938; Nooij and Mook, 1992; Knight et al., 2002). The toxic compounds therefore do not result in complete protection of the plant from nematodes, although symptoms from nematodes were described as minor (Nooij and Mook, 1992).

Live *P. lanceolata* plants reduced population levels of *M. xenoplax* on peach in microplot and greenhouse studies, but not to levels considered to be economically significant (Whittington and Zehr, 1992). Some of the reduction in nematode numbers may have resulted from competition between *P. lanceolata* and peach plants that reduced the peach root systems. The research did not investigate effects of plant extracts, so it is not known whether or not nematocidal compounds contributed to the suppressive effects of the plantain.

Ferris and Zheng (1999) investigated effects of *Plantago* extracts on *M. javanica* and *P. vulnus*. *Pratylenchus vulnus* individuals and *M. javanica* J2 were placed into water extracts prepared from *P. asiatica*. Similar to our studies with *P. lanceolata* and *P. rugelii* extracts, *P. vulnus* individuals became inactive and the effect was irreversible. However, *M. javanica* J2 activity decreased in the extracts but the nematodes recovered. This difference from our results may be due to the *Plantago* species tested, to variability in root-knot nematode species responses, or both. It is also notable that *M. javanica* was not affected by pH from 4.0 to 8.5 (Ferris and Zheng, 1999); the lowest pH value recorded for the *Plantago* extracts in our study was 4.62, which should not be a factor in nematode viability.

Our study demonstrated that extracts from *P. lanceolata* and *P. rugelii* were toxic to *M. incognita* but not to the tested bacteria and fungi. *Plantago lanceolata* shoot extracts tended to be the most active against *M. incognita* eggs and J2, making this species/plant part combination the leading candidate for further study of nematocidal compounds. However, the compounds found in both species have potential for use in selectively targeting plant-parasitic nematodes in pest management systems. Further research is needed to isolate and identify *Plantago*-specific compounds, to determine their toxicity to additional plant-parasitic nematodes, and to understand the fate of these compounds in soil.

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