A Protocol for Assessing Resistance to *Aphelenchoides fragariae* in Hosta Cultivars

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Abstract

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The use of resistant and tolerant cultivars is an important component of an integrated management plan for foliar nematodes on hosta. In order to identify tolerance and resistance in commercial hosta cultivars, reliable and efficient screening methods are required. To optimize the screening protocol, a series of greenhouse experiments was conducted using six hosta cultivars and two types of nematode inoculum. The pathogenicity and reproduction of *Aphelenchoides fragariae* maintained on fungal cultures versus maintenance on hosta were evaluated with two inoculation methods (with injury and without injury). Both sources of inoculum were pathogenic on all six cultivars tested but the

plant inoculum caused two to eight times larger lesions than the fungus inoculum. Both inocula caused larger lesions and resulted in higher reproduction rates on injured leaves than on noninjured leaves. Water soaking was more efficient than traditional Baermann funnel extraction methods. Correlations between foliage symptom severity and nematode reproduction were low or nonexistent. A numerical scale for faster assessment of disease severity was developed, and recommendations for a reliable protocol for assessment of resistance and tolerance are discussed.

Foliar nematodes *Aphelenchoides fragariae* (Ritzema Bos, 1890) Christie, 1932 (Aphelenchida: Aphelenchidae) are endo- and ectoparasites of many plants, including ornamental and agricultural crops (3,21). In nurseries and landscapes in the United States, these nematodes can be a serious problem (11–13) affecting hosta (*Hosta* spp.), a commonly grown herbaceous ornamental that thrives in shady environments. The nematodes can enter the foliar tissue through stomata or wounds (13,23) and feed on mesophyll cells (19,23), causing characteristic vein-delimited lesions that start as lightly chlorotic and then turn necrotic. The nematodes may overwinter in the soil, dormant crowns, and dry leaves (13). In temperate regions, they migrate to the new leaves in the spring (13).

Control of foliar nematodes on hosta can be difficult because of the survival behaviors of the nematode (3,13) and because hosta cultivars are popular perennial plants adapted to a wide geographical range, with numerous species and cultivars grown (20). Because thousands of plants are traded each year, there is increasing concern among growers about the movement of this nematode and fear of dissemination to noninfested areas. Forty-seven countries have legislation regulating the movement of this species in international trade (15). In order to develop adequate management strategies, it is important to effectively combine the knowledge of cultivar susceptibility with the existing chemical and cultural control options (11–14). However, there is currently no standardized method for assessing resistance of hosta cultivars to foliar nematodes. Jagdale and Grewal (13) tested the pathogenicity of *A. fragariae* on 23 cultivars, and found it pathogenic on 20 of them. They measured presence or absence of symptoms but did not assess symptom severity or nematode reproduction. Because the value of hosta plants lies mainly in the quality of their foliage, it is important to evaluate symptom severity. Symptom expression, however, can be highly variable due to influence of the environment and characteristics of the foliage of each cultivar. When

symptom development is slow, asymptomatic plants may support nematode reproduction (17). Consequently, nematode reproduction becomes an important part in the assessment of plant resistance and should be measured along with symptom severity. Breeders and growers would benefit from a standard protocol that assesses plant resistance and nematode reproduction (3).

The objective of this study was to develop a standardized protocol for the assessment of resistance to *A. fragariae* on hosta cultivars. In order to select the best procedures, we evaluated the effects of inoculum type (maintained on cultured fungus versus maintained on plants), inoculation method (with injury versus without injury), and nematode harvesting methods. We also explored potential correlations between nematode reproduction and symptom severity.

Materials and Methods

Hosta plants. Certified nematode-free hosta plants of six cultivars, representing a diversity of *Hosta* spp. commonly cultivated in the United States, were obtained from a commercial nursery in Spartanburg, SC. The cultivars selected included ‘Albo Marginata’ (*Hosta sieboldii*), ‘Aureo Marginata’ (*H. ventricosa*), ‘Fragrant Bouquet’ (*H. plantaginea*), ‘Golden Tiara’ (*H. nakaiana*), ‘Guacamole’ (*H. plantaginea*), and ‘Patriot’ (*H. sieboldiana*). Henceforth, we will refer to them by cultivar name only. The plants were grown in the greenhouse in individual pots until they had at least eight leaves. At least 30 plants of each cultivar were used to conduct these studies, according to the treatments described below.

Nematode inoculum sources. The effect of two types of *A. fragariae* inoculum (one maintained on a fungal culture [“fungus”] and one maintained on hosta plants [“plant”]) on pathogenicity, symptom severity, and host suitability on the six hosta cultivars was evaluated. The fungus nematodes are part of the Clemson University Nematode Collection and have been cultured in vitro on *Cylindrocladium* sp. grown in potato dextrose agar (HiMedia Laboratories) under laboratory conditions for 25 years. The origin of the isolate is uncertain. The plant nematodes were isolated from infected hosta in the South Carolina Botanical Garden, identified by morphology, and cultured on hosta in the field and the greenhouse. To obtain the fungus inoculum, nematodes were extracted by Baermann funnel (2) and a water suspension of mixed developmental stages from the extraction was adjusted to a concentration

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of 5,000 individuals per milliliter before inoculation. To obtain the plant inoculum, hosta leaves infected with *A. fragariae* were cut into 1-cm² pieces and soaked in water for 24 h. The mix was poured through nested sieves of 20 mesh (850 µm) and 500 mesh (25 µm) and the contents of the 500-mesh sieve were transferred to a Baermann funnel. The extract was washed several times with sterilized tap water and adjusted to the same concentration as the fungus inoculum. Each inoculum type was evaluated with each inoculation method (described below) on all six cultivars.

Leaf inoculation methods. Two inoculation methods (with and without leaf injury) were evaluated. Each combination of inoculum type and inoculation method was replicated on five plants of each of the six cultivars. In all treatments, nematode inoculation was conducted on two arbitrarily selected leaves of each plant. One leaf was not injured prior to inoculation and the other was injured by one of two methods: with a scalpel, by making five short cuts in the upper side of the leaf, or with a needle, by making 10 perforations scattered between the leaf veins. Both leaves were wrapped with wet tissue paper (Kimwipes, 11 by 21 cm; Kimberly-Clark) and 1 ml of the suspension of the nematodes was carefully dispensed on the tissue paper. The plants were covered with black plastic bags after inoculation in order to maintain a moist environment. The bags and tissue wrapping were removed after 72 h. All plants were kept in a shaded greenhouse at 25 ± 5°C. Treatments were arranged in a randomized complete block design and the experiment was performed twice.

Data collection. Inoculated hosta leaves were harvested 35 days after inoculation. Symptom severity was evaluated by calculating the percentage of affected leaf area using the grid method. Photographs of each inoculated leaf were taken and later used to aid in the development of a rating key. The leaves were cut into 1-cm²

pieces and soaked in tap water for 48 h at room temperature. The nematodes that emerged from the leaf pieces were recovered using nested sieves of 20 mesh (850 µm) and 500 mesh (25 µm) and counted.

Comparison of efficiency of extraction methods. Three harvesting methods were evaluated: traditional Baermann funnel, modified Baermann funnel, and water soaking. Symptomatic hosta leaves from different cultivars were collected, cut into 1-cm² pieces, and mixed together. Leaf pieces were equally divided among treatments and weighed before processing. The traditional Baermann funnel method was evaluated at room temperature (22 ± 2°C), whereas the modified Baermann funnel and water-soaking methods were evaluated at both room temperature and 28 ± 1°C. Three replicates were included for each treatment. The amount of nematodes recovered after 24 h was counted and recorded. For the traditional Baermann funnel method, leaf pieces were wrapped with tissue paper (Kimwipes, 11 by 21 cm) and placed in a glass funnel filled with tap water. For the modified Baermann funnel method, leaf pieces were wrapped with large Kimwipes (37 by 42 cm) and placed on a 20-mesh sieve (25 cm in diameter, 850-µm openings). The sieve was placed in a plastic container with tap water just covering the leaf material. A small aquarium pump was used to aerate the water during the incubation. For the water-soaking method, leaf pieces were placed in petri dishes (10 cm in diameter) filled with tap water. The nematodes that emerged from the leaf pieces were recovered using nested sieves of 20 mesh (850 µm) and 500 mesh (25 µm) and counted.

Statistical analysis. All nematode density data were natural log-transformed, except for those for the extraction method experiment. Nematode density, symptom severity, and extraction efficiency data were analyzed by one-way analysis of variance with

Fig. 1. Effect of *Aphelenchoides fragariae* inoculum type (maintained on fungus or plants) on nematode density on six hosta cultivars: 'Albo Marginata', 'Fragrant Bouquet', 'Golden Tiara', 'Guacamole', 'Patriot', and 'Aureo Marginata'. Error bars are standard error of the mean ($n = 20$). Different letters indicate significant ($P < 0.05$) differences within the same cultivars according to Student's t test.

Table 1. Severity of symptoms, measured as percentage of leaf area with lesions, caused by *Aphelenchoides fragariae* maintained on hosta (plant inoculum) versus *Cylindrocladium* spp. (fungus inoculum) on four hosta cultivars ($n = 10$)

Type of inoculum	Leaf area with lesions (%) ^a			
	Albo Marginata	Aureo Marginata	Guacamole	Patriot
Plant inoculum	10.34 ± 1.79 A	4.17 ± 1.51 A	25.95 ± 0.85 A	10.34 ± 3.43 A
Fungus inoculum	4.13 ± 0.75 B	1.21 ± 0.67 B	3.53 ± 0.71 B	1.22 ± 0.55 B

^a Different letters indicate significant differences ($P < 0.05$) within the same column (cultivar) according to Student's t tests.

JMP 9 software (SAS Institute). Differences between treatments were determined by Fisher's least significant difference or Student's t test at $P < 0.05$. Potential correlations between symptom severity and nematode density were analyzed using the Bivariate fit procedure of JMP 9 software. If there were no differences between repeated experiments ($P > 0.05$), data were combined for analysis.

Results

There was no effect of the separate trials ($P > 0.05$); therefore, the results for each experiment are presented based on combined trials.

Effect of inoculum source. Both types of inoculum (plant and fungus) were pathogenic to all six cultivars tested but there were

Fig. 2. Effect of injury on *Aphelenchoides fragariae* density following application of two types of inoculum (maintained on fungus or plants). Error bars are standard error of the mean ($n = 44$). Different letters indicate significant ($P < 0.05$) differences within the same inoculum type according to Student's t test.

Fig. 3. Comparison of five extraction methods for recovery of *Aphelenchoides fragariae* individuals from hosta (*Hosta* spp.) leaves. Error bars are standard errors of the means. Extraction methods not sharing a common letter indicate significant ($P < 0.05$) differences according to Fisher's least significant difference ($P < 0.05$).

significant differences ($P < 0.05$) between the two types of inoculum sources in nematode reproduction and disease severity (Fig. 1; Table 1). Disease severity, measured as percentage of the leaf area with lesions, was higher in leaves treated with the plant inoculum (Table 1). Characteristic, vein-delimited lesions were observed as early as 21 days after inoculation and progressed from yellow to brown in color as time passed. Overall, lesions were two to eight times larger with plant inoculum than with fungus inoculum. The largest differences in disease severity as a result of inoculum type were observed with Patriot, where lesions were about 10% of leaf area with plant inoculum and about 1% with fungus inoculum. The disease severity data for Fragrant Bouquet and Golden Tiara were not included in Table 1 because the occurrence of other foliar dis-

ease symptoms compromised the reliability of the symptom severity evaluation. However, we did include the nematode density data for all six cultivars. On five of the six cultivars, nematode reproduction was higher in the leaves inoculated with plant inoculum (Fig. 1). Only Albo Marginata supported higher nematode densities per leaf with the fungus inoculum (Fig. 1). There were differences ($P < 0.05$) between nematode densities obtained with plant inoculum and with fungus inoculum on Albo Marginata, Golden Tiara, Guacamole, and Patriot. For Fragrant Bouquet and Aureo Marginata, there were no statistical differences between the plant and fungus inocula. The highest nematode reproduction was observed on Aureo Marginata (17,400 individuals/leaf recovered 35 days after inoculation), using plant inoculum.

Effect of inoculation method. There were differences ($P < 0.05$) in nematode reproduction and disease severity between inoculation treatments with and without injury (Fig. 2; Table 2). Symptom severity of leaves injured at the time of inoculation was greater when observed 35 days after inoculation. The difference in disease severity was more noticeable with plant inoculum than with fungus inoculum. The combined mean size of the lesions was doubled for fungus inoculum when aided by injury but was more than eight times larger for plant inoculum when aided by injury. Nematode reproduction was enhanced in a similar manner (Table 2). When combining the data for all cultivars, nematode densities were up to 38-fold higher on injured leaves with plant inoculum, and up to 3.7-fold higher on injured leaves with fungus inoculum.

Comparison of efficiency of extraction methods. Numbers of nematodes extracted from the same weight of leaf material were different ($P < 0.05$), depending on the extraction method used (Fig. 3). The traditional Baermann funnel technique yielded the lowest number of individuals but it was the most consistent technique (lowest standard deviation), and the extracts also contained the least leaf debris. This is important because it makes the nematodes within the extracts easier to quantify. The most efficient extraction method tested was water soaking, which yielded more than four times more nematodes than were obtained with the traditional Baermann funnel technique. Incubation temperature did not change the efficiency of the water-soaking or the modified Baermann funnel techniques. The modified Baermann funnel technique yielded extracts with the most debris and, consequently, was the most difficult to quantify.

Correlation between disease severity and nematode density. For three of four cultivars, the severity of symptoms increased directly with nematode population but the level of correlation varied by cultivar (Fig. 4). For Albo Marginata, nematode population density and disease severity were not correlated. A low positive correlation ($r = 0.538$; $P < 0.05$) between these two variables was observed for Guacamole. Higher positive correlations were observed with Aureo Marginata ($r = 0.734$; $P < 0.05$) and Patriot ($r = 0.856$; $P < 0.05$). Equations were derived to describe relationships between population density of *A. fragariae* and symptom severity for the three cultivars with positive correlations (Fig. 4). In each, the slope of the line is a measure of the susceptibility of the cultivar. The most susceptible cultivar (i.e., the one with the largest lesions caused by a given number of nematodes) was Guacamole.

Fig. 4. Regression analysis of symptom severity (expressed as percentage of leaf area with lesions) caused by *Aphelenchoides fragariae* versus nematode density on three *Hosta* cultivars: A, 'Aureo Marginata'; B, 'Guacamole'; and C, 'Patriot'.

Table 2. Severity of symptoms, measured as percentage of leaf area with lesions, caused by *Aphelenchoides fragariae* maintained on hosta (plant inoculum) versus *Cylindrocladium* spp. (fungus inoculum) and inoculated on leaves with and without mechanical injury^y

Inoculation method	Leaf area with lesions (%) ^z	
	Plant inoculum	Fungus inoculum
Injured	22.53 ± 5.76 A	3.23 ± 0.52 A
Noninjured	2.68 ± 0.76 B	1.60 ± 0.47 B

^y Data for six cultivars were combined ($n = 30$). Injuries were made with a needle (10 perforations per leaf).

^z Different letters indicate significant differences ($P < 0.05$) within the same column (inoculum type) according to Student's *t* tests.

Discussion

The use of resistant and tolerant cultivars is an important component of an integrated management plan for foliar nematodes on hosta. In order to identify tolerance and resistance in commercial hosta cultivars, reliable and efficient screening methods are required. Based on the results of the experiments of this study and on our experience working with this nematode, we recommend the following protocol for screening for resistance to foliar nematode on hosta cultivars. Maintain and increase foliar nematode inoculum on hosta plants in the greenhouse. We recommend Patriot and Guacamole for this purpose, because they are commonly grown cultivars that sustain adequate nematode reproduction. For inoculum extraction, cut infected hosta leaves into 1-cm² pieces and soak in tap water for 48 h at room temperature. Pour the mix through nested sieves of 20 mesh (850 µm) and 500 mesh (25 µm). Wash contents collected on the 500-mesh sieve with tap water. Adjust the concentration of the extract to 5,000 mixed stage individuals per milliliter and use within 3 days of extraction. Hosta plants to be evaluated should be potted individually, and each plant to be inoculated should have at least eight leaves. Select two healthy leaves on each hosta plant and make 10 perforations, scattered between leaf veins, with a needle on both leaves. Wrap both leaves with wet

tissue paper (e.g., Kimwipes, 11 by 21 cm). Dispense 1 ml of the suspension of nematodes on one leaf, and 1 ml of sterile tap water on the other leaf as negative control. Cover the plants with black plastic bags. After 72 h, remove the bags and tissue wrappings. Use a completely randomized arrangement for the experimental design with at least five replications. Run the experiment at least twice. One susceptible cultivar should be included in each experiment as positive control. Based on the results of this study, we recommend Guacamole as the susceptible control cultivar. Maintain the greenhouse at 25 ± 5°C and use shade cloth (50 to 60% density). Water the plants carefully to avoid splashing. Provide a light-and-dark phase of 12 h, supplementing with artificial lights when necessary.

After 35 days post inoculation, collect inoculated leaves and assess symptom severity of each, using a 0-to-6 scale (Figs. 5 and 6) based on the percentage of the total leaf area with lesions and chlorosis. Evaluate the positive and negative controls first. If the positive control is rated 0 or 1, discard the test and run again. If the negative control (i.e., the leaves inoculated with water) is not rated 0, discard the test and run again. Cut inoculated leaves into 1-cm² pieces and soak in a 10-cm-diameter glass petri plate with 40 ml of tap water for 48 h at room temperature. Retrieve the nematodes passing through nested sieves of 20 mesh (850 µm) and 500 mesh (25 µm) and count. Report the numerical rating for symptom

Fig. 5. Rating chart of symptom severity on hosta caused by foliar nematode (*Aphelenchoides fragariae*). Drawings of hosta leaves are based on appearance of 'Guacamole' hosta.

severity and the nematode density data for each plant and cultivar.

Variability in rates of reproduction of *A. fragariae* in experimental culture is often high (3). There have been very few studies on the fate of nematodes immediately after inoculation but loss is believed to be very high. Plowright and Gill (18) estimated that more than 75% of *Ditylenchus angustus* inoculum was lost after inoculation. Our observations support the use of wet tissue and plastic bags for minimizing inoculum loss, although we have simply observed improved infection and have not quantified losses. These observations are also supported by the fact that a common cultural control recommendation for foliar nematodes is to avoid excess surface moisture of the foliage (21).

It is convenient to rear nematodes in pure cultures either in fungal cultures or on callus tissue. Several migratory endoparasitic

nematodes such as *Pratylenchus* spp., *Radopholus* spp., and *Ditylenchus* spp. are routinely cultured on carrot disks or using other in vitro methods. Some authors report changes in infectivity of the inoculum reared in vitro (22) while others report no differences (5,10). Ali and Ishibashi (1) recommend that infectivity and aggressiveness of *Ditylenchus* spp. reared in monoxenic culture be monitored on the field host. In contrast, Erikson (6) reports having successfully maintained and regularly subcultured a lucerne race of *D. dipsaci* on callus for more than 10 years.

In our studies, *A. fragariae* lost virulence when maintained on fungal cultures. Researchers who, for convenience, decide to use inoculum reared on fungi should be aware that symptom expression and nematode reproduction can be greatly reduced. Additionally, culturing nematodes on fungi comes with the risk of inoculating plants with fungal propagules along with the nematode inoculum (16). This is an important risk if the fungal host is patho-

Fig. 6. *Hosta* (*Hosta* spp.) leaves illustrating the different degrees of foliar nematode (*Aphelenchoides fragariae*) symptom severity, according to our suggested rating chart. A, Leaf of 'Guacamole', rated 0. B, Leaf of 'Golden Tiara', rated 1. C, Leaf of 'Patriot', rated 2. D, Leaf of 'Green Piecrust', rated 3. E, Leaf of Guacamole, rated 4. F, Leaf of Guacamole, rated 5.

genic to plants; for example, *Rhizoctonia solani* (13), *Botrytis cinerea* (3), and *Cylindrocladium* sp. (this study).

Wingfield (24) observed that races of *Bursaphelenchus xylophilus* with low pathogenicity to pine could be more vigorous in the mycophagous phase. *A. fragariae* is closely related to *B. xylophilus* and similar to it in its ability to feed on both fungi and plants (3,13,21); however, distinct phytophagous and mycophagous phases of the foliar nematode life cycle have not been characterized. Fungal feeding in foliar nematodes is believed to play an important role in survival in the soil and plant debris, and it is possible that isolates with relatively low virulence are more vigorous when feeding on fungi than when feeding on plants. This hypothesis is yet to be tested.

We recommend injuring hosta leaves with a needle as part of the inoculation procedure. Our research showed that foliar nematodes will infect hosta plants without the aid of injury but that certain cultivars will show few to no symptoms without injury. Jagdale and Grewal (13) also demonstrated that injury was necessary to cause symptoms on cultivars such as 'Fried Green Tomatoes' and 'Fragrant'. In our study, we found the same was true for Patriot. We recommend small perforations (>1 mm) made with a sharp needle. In the control mock inoculations with water, these perforations were healed by 35 days. For cultivars with more succulent leaves, injury with a scalpel (cuts) should be avoided, because we observed that these are readily colonized by opportunistic fungi. Because injury cannot be completely avoided in commercial settings (transporting, cultural practices, and so on) or in nature (wind, insects, and so on), we believe that injury should be part of the standard protocol.

We recommend the water-soaking method at room temperature for extraction of foliar nematodes from leaf tissue. This method is simple and yielded adequate amounts of nematodes free from leaf tissue debris. The traditional Baermann funnel technique yielded a cleaner extract and also required little labor and simple equipment but the extraction efficiency was lower. Fortuner (7) reported that specimens can be trapped by the tissue and the sides of the funnel with this extraction method. We were expecting the extraction efficiency to be improved by aeration (modified Baermann), because oxygenation of the water has been reported to improve migration of nematodes from the plant tissue (8,9), but we did not observe such improvement. It is possible that aerating favored the growth of fungi and bacteria that could have affected nematode motility.

Differences in host response and nematode reproduction between series of experiments conducted under similar conditions are not uncommon in nematology (4). Such differences may arise from differences in the environmental conditions, developmental stage of plants, and infectivity of the inoculum. The occurrence of these differences underlines the importance of including the same susceptible control host in each experiment. This way, comparison of results obtained from different tests is possible.

The variability of correlation between symptom expression and nematode reproduction in our studies indicates the importance of measuring both parameters when evaluating cultivars. Our suggested protocol assesses both resistance (reduced nematode reproduction) and tolerance (reduced loss of aesthetic value caused by symptom expression, regardless of nematode reproduction), and provides a useful tool for breeders, nematologists, consultants, and extension specialists in the development of new cultivars and of recommendations regarding cultivar selection.

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