ABSTRACT

MCCUISTON, JAMIE L. Development of a PCR-Based Diagnostic Assay and Sampling Protocol for the Detection of *Aphelenchoides fragariae* in Ornamental Host Crops. (Under the direction of Dr. C.Y. Warfield and Dr. E.L. Davis.)

Three foliar nematode species, *Aphelenchoides fragariae*, *Aphlenchoides ritzemabosi*, and *Aphelenchoides besseyi* are pathogens of economic importance in the ornamental horticulture industry within and outside the United States. These microscopic worms thrive in greenhouses and nursery conditions due to their ability to infect over 250 species of ornamental foliage plants, as well as a large number of perennials, bedding, foliage, and woody ornamentals. They cause chlorotic and necrotic lesions that typically follow the venation pattern of the leaf. Plant symptoms generally do not appear until the plant is heavily infected with nematodes, which makes early detection problematic. As a result, nematode-infected plant materials are being unknowingly propagated and moved across nursery state and national boundaries into previously uninfested nurseries, resulting in millions of dollars of plant damage each year. The traditional water extraction method of foliar nematode detection requires 24-48 hours for the nematodes to migrate out of plant tissue samples into surrounding water for subsequent microscope identification. The water extraction assay is cumbersome, time-consuming, requires trained personnel for foliar nematode identification, and has limited sensitivity in plant samples that harbor low populations of foliar nematodes. A PCR-based diagnostic assay was developed for the detection of *A. fragariae* using species-specific primers (AfragF1 and AfragR1) developed inside the unique ITS1 region of the ribosomal DNA. DNA sequences from seven *A. fragariae* populations, a known population of *A. fragariae*, and ITS1 sequence available in GenBank (Accession number: AF119049) (Iwahori et al., 1998) were compared and found to

be 100% identical to the sequence amplified using the AfragF1/AfragR1 primers. The PCR

assay was specific to only *A. fragariae* based upon comparisons with ITS1 sequences of *A.*

ritzemabosi and *A. besseyi*, and the primers also failed to amplify DNA of any host plant

tested nor common greenhouse fungal, bacterial, insect, and mite pests. The PCR-based

assay was highly sensitive; one *A. fragariae* nematode could be detected in up to 146 mg of

background leaf tissue. *Aphelenchoides fragariae* were detected by PCR in all of 112 plant

samples collected, with only 93 of the samples yielding specimens by water extraction that

were identified morphologically as *A. fragariae*. The PCR-based assay detected significantly

more ($p < 0.05$) more nematodes in asymptomatic leaf tissue as compared to the water

extraction assay. For leaves with visual symptoms of foliar nematode infection, both

methods were statistically equivalent in detecting nematodes in leaf tissue. Both the PCR

assay and the water extraction technique were compared in split samples to assess foliar

nematode distribution within plant architecture and within blocks of different plant species

grown in commercial nurseries. Although foliar nematodes were detected in all parts of

Verbena 'Snowflurry' plants, the majority of the nematodes were located in the lower third of

the plant. The *A. fragariae* PCR assay was significantly more sensitive than the water

extraction assay in detecting foliar nematode infection of plants in separate blocks of

Anenome spp., *Disporum smilacinum* and *Oxalis regnellii,* but heavy infection of a block

Mildella nidulata plants by foliar nematodes did not indicate a difference between detection

assays. Application of Taylor's Power Law on nematode counts from water extraction assays

of the *D. smilacinum, O. regnellii, and M. nidulata* plant blocks suggested a mostly

aggregated distribution of foliar nematodes within each block and optimal sampling sizes of

23, 38, and 41 plants depending upon plant species. Coupling of the increased sensitivity and

specificity of the PCR-based assay with assessment of nematode distribution within plants

and nursery facilities will provide an improved instrument for management decisions to

reduce foliar nematode damage to ornamental nursery plants.

DEVELOPMENT OF A PCR-BASED DIAGNOSTIC ASSAY AND SAMPLING PROTOCOL FOR THE DETECTION OF *APHELENCHOIDES FRAGARIAE* IN ORNAMENTAL HOST CROPS

By

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DEDICATION

I dedicate this thesis to my parents, James and Robin McCuiston for their love, patience, support, understanding, and teaching to make me the person I am today. As well as, Anthony Keene, for his love, understanding, devotion, and patience during the long days and nights it has taken to complete this project.

BIOGRAPHY

Miss Jamie Leigh McCuiston was born in Greensboro, North Carolina on a cool morning March 26, 1982. She was raised on a family farm specializing in beef production located in Summerfield, NC where, in addition to the cattle, a few goats were maintained to provide milk to Jamie and her brother when they were infants. She was a proud class of 2000 graduate from Northwest Guilford High School and received her Bachelor of Science Degree with honors from North Carolina State University concentrating in biological sciences with a minor in genetics. She was an active member of several honors societies including Alpha Zeta where she learned the importance of agriculture in everyday life. She elected to continue her education at NC State by pursuing a Masters Degree in Plant Pathology. She plans to take the knowledge obtained in lab techniques to the business to make a positive difference in the future of agriculture in today's world. Jamie lives in the Raleigh area with the love of her life and her best friend, Anthony and their two dogs, Molly and Junior.

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I would also like to express my deepest thanks to my parents, James and Robin McCuiston; my brother, Cody; for their unconditional love, confidence, and support throughout my life to make me the person that I am today, I love all of you very much. Finally, I want to extend a special thank you to the love of my life, Anthony Keene, who has been with me since I began my Master's Project and has provided me with unconditional love, support, devotion, and for being a good listener throughout the easy and the tough times in my project. I love you very much.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ix

INTRODUCTION

The genus *Aphelenchoides* contains over 197 published species of nematodes
(Maggenti, 1981). Species of *Aphelenchoides* are either saprophytic in the soil and water,
plant-parasitic, or associated with insects (Sanwal, 1961). The most important species in
ornamental and foliage plant production are *Aphelenchoides fragariae* and A*phelenchoides
ritzembosi*; however, *Aphelenchoides besseyi* has also been reported on a few ornamental
hosts. (Jenkins and Taylor, 1967). These species are associated with aboveground plant
parts, therefore the nematodes are commonly termed foliar, leaf, or bud nematodes. The
mode of infection, feeding, and plant symptoms caused by these three foliar nematode
species are similar, however, the range of host plant species varies widely across
Aphelenchoides species (Maggenti, 1981).

Foliar nematodes are found throughout the world on almost every continent. *A.
besseyi* infects rice and strawberries but has also been reported to infect a limited number of
ornamental plant species (Jenkins and Taylor, 1967). Due to *A. besseyi's* host range, the
nematodes have been reported on the continents of Africa, Asia, Australia, Europe, and
North and South America (Hunt, 1993). *A. ritzemabosi,* commonly known as the
chrysanthemum nematode, has a host range of almost 200 different plant species including
Chrysanthemum, Anemone, Iris, Dahlia, Verbena, and others (Decker, 1989). *A. ritzemabosi*
has been mainly reported in the cool, moist areas of the temperate zone (Decker, 1989). This
species has been detected in Asia, Europe, North and South America (Jenkins and Taylor,
1967, Vovlas et al., 2005, Klinkenberg, 1955, Hunt, 1993). *A. fragariae* is commonly known
as the strawberry nematode due to its infection strawberries. *Aphelenchoides fragariae* has a
host range of over 250 host plants in 47 families. Plants susceptible to *A. fragariae* include

Ruscus, *Rieger* begonia, *Anthurium*, *Hosta*, and others (Mor and Spiegel, 1993, Riedel and Powel, 1975, Hunter et al., 1974, and Noel, 1994). *Aphelenchoides fragariae* have been reported primarily in moderate or temperate climate zones of Asia, Australia, Europe, and North and South America (Christine, 1959; USDA, 1978). *A. besseyi*, *A. ritzemabosi*, and *A. fragariae* have not only overlapping geographical distributions, but similar morphology (Siddiqi, 1975; Hunt, 1993) making the identification of each species difficult.

As members of the order Aphelenchida, all foliar nematodes are slender, vermiform, and have a strongly developed muscular metacarpus that expands almost the entire width of the body (Hunt, 1993). *Aphelenchoides besseyi* has a finely annulated body with a rounded, unstriated, and slightly offset cephalic region (Siddiqi, 1975). The nerve ring of *A. besseyi* is located about one body width behind the median bulb and the excretory pore is located near the anterior edge of the nerve ring. The females have short ovaries with two to four rows of oocytes. The female tail terminus has three to four pointed mucro. The males have spicules that lack an apex and has a single outstretched testis. The male tail is conoid with two or four pointed mucro. *Aphelenchoides fragariae* have a cephalic region that is smooth and elevated with sides straight to rounded almost continuous with the body (Siddiqi, 1975). The body of *A. fragariae* has two lateral lines running the length of the body. The excretory pore is level with or close behind the nerve ring, which is located about one body width behind the medium bulb. The tail is elongate ending in a simple blunt spike without any mucro. The female ovaries are outstretched, with a single row of oocytes. The males have outstretched testis with spermatocytes in a row. The spicules are rose-thorn shaped with a moderately developed apex. *Aphelenchoides ritzemabosi* has a hemispherical cephalic region that is offset by a constriction that is slightly wider than the body (Siddiqi, 1974; Steiner, 1932).

The body of *A. ritzemabosi* has four lateral lines running the length of the nematode. The excretory pore is level with or close behind the nerve ring, which is located about one body width behind the median bulb. The tail is elongate ending in a terminus with two to four mucro. The female ovaries are outstretched with multiple rows of oocytes. The males have an outstretched genital tract with a single testis. The morphological characteristics that distinguish *A. besseyi*, *A. fragariae*, and *A. ritzemabosi* are difficult to resolve using a light microscope and require a trained eye.

The infection and symptomology of *A. fragariae* and *A. ritzemabosi* on ornamental plants have been more extensively studied than that of *A. besseyi* on similar plants. *Aphelenchoides besseyi* infects the seeds and leaves of rice and strawberries causing white tip disease of rice and summer dwarf disease of strawberry (Christie, 1932). The leaf tips of rice may be white or yellow which will become brown or black as the degree of infection increases. The rice grains are typically shorter, twisted, and wrinkled, causing a delay in maturity ultimately decreasing yield (Christie, 1932; Todd and Atkins, 1958; Yokoo, 1948). *Aphelenchoides besseyi* infects the bulb and leaves of ornamental plants causing similar symptoms as that of infected strawberry plants (Plakidas, 1928). The leaves of infected strawberry and ornamental plants appear unsymmetrical, brittle, and crinkled with cupped margins. *Aphelenchoides besseyi* live in the seeds and bulbs causing no apparent signs of infections until the seed or bulbs sprout and the nematodes move to the leaves and feed causing symptoms (Thorne,1961). Like *A. besseyi*, *A. fragariae* is also able to infect strawberries and has a wide range of ornamental plant host species. *Aphelenchoides fragariae* was first observed in 1889 on strawberry plants in England (Decker, 1989). Due to this first report, the species *A. fragariae* was given the common name the strawberry

nematode, but has since been found to cause serious damage to multiple ornamental plant species (Grewal and Jagdale, 2001). *Aphelenchoides fragariae* can infect more than 250 plant species in 47 families (Decker, 1989). The nematodes are believed to overwinter as juveniles and adults in the soil and dormant bulbs, when the plants break dormancy in early spring, the nematodes begin to migrate in thin films of water to the leaves (Jagdale and Grewal, 2006, Wallace, 1959). The nematodes are able to enter the leaves through open stomata, through direct contact between healthy and infected leaves, or through wounds. (Jagdale and Grewal 2006; Wallace,1959). Research has been done to determine if a stimuli is present to signal infection by foliar nematodes (Klingler, 1970) using a thin film of water over plastic foil with micro-openings to simulate stomata. Carbon dioxide was forced through the micro-openings in the presence of foliar nematodes to simulate gas exchange through a leaf. Foliar nematodes rapidly accumulated at the openings and entered the openings, in contrast to very few nematodes that found and entered the openings in the absence of CO_2. It is unknown, however, if other factors aid in the infection process of foliar nematodes (Klingler, 1970). Upon entry into the leaf the nematodes feed upon the mesophyll cells but are unable to cross the veins in the leaf, creating a brown angular lesion (Wallace, 1959 and Sanwal, 1959). The infected host plant releases polyphenols from conjugated phenols through the action of enzymes such as beta-glycosidase causing the browning of infected tissue, which is not present in healthy tissue (De Meseneer, 1964). Foliar nematodes usually complete their life cycle inside the leaves. At an optimum temperature of 18°C eggs are laid in the mesophyll where the juveniles hatch and develop into sexually mature adults within 10 to 11 days (Decker, 1989). A heavy infection can occur rapidly if the conditions remain favorable for multiple generations of nematode reproduction within leaf tissue (Decker,

4

1989).   In order for the nematodes to cross the veins within the leaves, the nematodes must exit the leaves through the stomates and migrate in thin films of water to a different part of the leaf, reinfecting again through the stomates.

The foliar nematode *A. ritzemabosi* has a similar biology, symptomology, feeding habits, and host range to that of *A. fragariae*. *Aphelenchoides ritzemabosi* is has been reported to infect almost 200 plant species and is an important disease in ornamental chrysanthemums (Jenkins and Taylor, 1967). *Aphelenchoides ritzemabosi* overwinters in the soil and dead infected leaves, however, the main means of spread is through infested plant propagative cuttings (Stewart, 1921). During the summer months, the nematodes become active, migrating up the surface of stems in a think film of water to infect the leaves in buds (Wallace, 1959). The adults are able to migrate upward against a downward flow of water but the juveniles are only able to move in still water (Wallace, 1959). Upon reaching the leaf tissue *A. ritzemabosi* enter through the stomata and live in the intercellular spaces, where they feed on the mesophyll cells. The cells begin to break down causing the leaf to turn brown (Wallace, 1961). The true cause of the leaf browning is unknown but it is thought that the phenolic acids, chlorogenic acid and isochlorogenic acid, are metabolized resulting in oxidation and polymerization causing the production of brown pigments (Wallace, 1961). Unfortunately, the symptoms of *A. ritzemabosi* infection are either not visible or mistaken for other pathogens during early infection making diagnosis and control difficult (Vovlas et al., 2005). *Aphelenchoides ritzemabosi* can complete an entire life cycle in 11 to 14 days at a temperature range of 14°C to 17°C (Stewart, 1921). In one growing season as many as ten generations of foliar nematodes can occur, creating more inoculum to be spread throughout the nursery (Jenkins and Taylor, 1967)

Management of foliar nematodes in ornamental plant nurseries is limited because the symptoms are often difficult to diagnose. By the time an infection is detected the nematodes may have spread to neighboring plants and possibly to different parts of the nursery through contact with infected foliage or in irrigation water. The best management strategy for foliar nematodes is exclusion, using nematode-free planting stock, propagation materials, and cultural practices (Jenkins and Taylor, 1967). Once established within a planting, control of foliar nematodes is very difficult due to the lack of effective chemical or biological methods. Several nematicides, insecticides, and acaracides including oxamyl, parathion, abamectin, diazinon, and methiocarb are effective in foliar nematode suppression, however the US Environmental Protection Agency (EPA) has banned or restricted the use of these pesticides for foliar nematode control (Jagdale and Grewal, 2002, 2004; Johnson and Gill, 1975; La Mondia, 1999). The bans and restrictions are due to environmental pollution concerns and/or human health risks (Schulze, 2003). A hot water dip of infected plant material has been tested and used on a limited basis as an alternative form of treatment for foliar nematode control (MacLachlan and Duggan, 1979; Qui et al., 1993; Riedel and Powers, 1974; Yamada and Takakura, 1989). Bare-rooted plants, dormant crowns, bulbs, and runners of plants that are of economic importance in the ornamental horticulture industry are routinely immersed in hot water for the control of foliar nematodes (Jagdale and Grewal, 2004). The use of a hot water dip for the control of foliar nematodes is time-consuming, labor intensive, and requires equipment to control the temperature of the water. The dip is also difficult for homeowners that have infected plants (Jagdale and Grewal, 2004). Therefore, a hot water drench was also evaluated for the effectiveness of foliar nematode control in the soil or on the leaves or fronds of *Hosta* (sp.) and ferns (*Matteuccia pensylvanica*) to reduce overwintering *A.*

fragariae populations (Jagdale and Grewal, 2004). Foliar nematode populations were reduced in the soil using three treatments of water between 44.4°C to 58°C. The populations were also reduced in Hosta leaves and fern fronds using three monthly treatments of 100°C water. The ferns showed no adverse effects to the hot water drench treatments. The *Hosta* exhibited no adverse effects for the first two hot water drench treatments, however the *Hosta* plants exhibited a reduction in leaf size and number after the third hot water drench treatment (Jagdale and Grewal, 2004). Additionally, many other ornamental plants are sensitive to the excessive temperatures and length of time the hot water drench is necessary to kill foliar nematodes (Jagdale and Grewal, 2004). Therefore, hot water treatments are not practical for the control of foliar nematodes in all plant species.

Due to the lack of adequate treatment measures the most important control strategy to reduce foliar nematode damage is the development of ways to minimize nematode introduction and spread into the nursery or greenhouse. For long-term control it is necessary to establish good sanitation practices throughout the greenhouse or nursery to minimize introduction or spread of foliar nematodes. Good nursery sanitation practices to reduce foliar nematode infestation include minimizing leaf wetness, removing leaf litter throughout the greenhouse, sanitizing cutting utensils, appropriate hand washing, and floor sanitation (Jenkins and Taylor, 1967). If a foliar nematode-infected plant is found either through visual assessment or laboratory analysis, the infected plant and the plants immediately surrounding it should be removed and destroyed. The area should then be sanitized using a 10% bleach solution and allowed to dry completely before nematode-free plants are reintroduced.

Diagnosis of foliar nematode infection of plants is a specialized skill that is usually conducted on samples submitted to a professional laboratory. Foliar nematode infection can

be difficult to detect because of a lack of symptoms upon initial infection of host plants or if symptomatic tissues of an infected plant have been pruned. Therefore, an appropriate sampling strategy coupled with an accurate, sensitive, and efficient detection assay is needed to better manage the spread of foliar nematodes among host plants. Current methods to detect and identify foliar nematodes in samples of plant foliage include a Baermann funnel with or without mist extraction, and a water incubation technique (Whitehead and Hemming, 1965; Jagdale and Grewal, 2002). These extraction techniques are all based on the ability of foliar nematodes to migrate out of plant samples into collecting water (Jenkins and Taylor, 1967). For the Baermann funnel mist extraction, the leaf specimens are placed in moist conditions allowing the nematodes to crawl or swim out of the foliage and be collected in water (Whitehead and Hemming, 1965). The water incubation extraction of foliar nematodes also relies on ability of the nematodes to migrate out of the tissue during incubation in a small volume of water for 24 to 48 hours at room temperature (Jagdale and Grewal, 2002; Vovlas et al., 2004). Upon extraction of the foliar nematodes out of plant samples by either method, the nematodes are identified under a microscope using morphological characteristics unique to each species. The limitations of the current methods include reliance on active nematode migration out of plant samples, specialized training to identify nematodes using morphology, and extractions that require 24 to 48 hours to complete. Saprophytic nematodes also have the ability to live on the leaf surface and may end up in the extraction water along with any foliar nematodes from infected foliage, further complicating accurate identification. Foliar nematodes may not be recovered from plant samples that harbor low populations of nematodes using the conventional extraction methods, presenting problems with assay sensitivity.

The use of polymerase chain reaction (PCR) to specifically amplify the internal transcribed spacer regions (ITS1 and ITS2) of ribosomal DNA (rDNA) from individual nematodes provides a potential means to identify nematodes without bias or special training from small samples and potentially from asymptomatic infected plants (Powers et al., 1997). The eukaryotic ITS region is flanked on either side with the 18S and 28S ribosomal DNA subunit. The 18S, 5.8S, and 28S rDNA subunits are coded rRNA regions, which are minimally variable across species because of the selection pressure imposed by their role in translation (Powers et al., 1997). The ITS regions of rDNA, however, are noncoding regions free of strong selection pressure and therefore more variable. The variability of the rDNA ITS regions can be used for comparison of species (Iwahori et al., 1998). Nematologists have used the ITS region to identify species of plant-parasitic, animal-parasitic, and insect-parasitic nematodes (Beckenbach et al., 1999; Cherry et al., 1997; Chilton et al., 1995; Fallas et al., 1996; Fallas et al., 1994; Harmey and Harmey, 1993; Ibraham et al., 1994; Joyce et al., 1994; Nasmith et al., 1996; Powers and Harris, 1993; Powers et al, 1997; Stevenson, 1995; Szalanski, 1997; Thiery and Mugniery, 1996, Vrain et al., 1992; Vrain and McNamara, 1994; Wendt et al. et al., 1995; Zijlstra et al., 1997; Zijlstra et al., 1995). "Universal" PCR primer sets located in the flanking, conserved rDNA subunit regions have been used to obtain and identify the ITS1and ITS2 rDNA regions in the absence of known sequence (Powers et al., 1997). Powers et al. (1997) have stated that to their knowledge, "universal" primer sets have not failed to produce an ITS rDNA PCR product for any single nematode species tested. The ability to specifically amplify the ITS region from an individual nematode species suggests that any species, population, or community of nematodes can be analyzed both qualitatively

and quantitatively using a molecular assay based on the ITS region (Vrain and McNamara, 1994).

A decline in emphasis and specialists within the field of classical taxonomy combined with advances in molecular phylogeny has prompted the development of molecular diagnostic tools for nematodes (Coomans, 2002; Oliveira et al., 2005). Diagnostic protocols need to be objective, sensitive, accurate, reproducible, rapid, user-friendly, and not require the expertise of a trained taxonomist (Oliveira et al., 2005). Molecular diagnostic assays have been developed for different species of plant-parasitic nematodes within the genera *Heterodera*, *Globodera*, *Bursaphelenchus*, *Meloidogyne*, *Pratylenchus*, and *Xiphinema* (Iwahori et al., 1998; Sheilds et al., 1996; Subbotin et al., 1999, 2000; Uehara, 1998; Oliveira et al., 2005; Wang et al., 2003; Zijlstra et al., 1995). Although a molecular diagnostic assay for foliar nematodes does not currently exist, *A. fragariae* was used as a control species of Aphelenchida in reference to phylogenetic analysis and diagnosis of *Bursaphelenchus* nematodes using RFLP and rDNA sequence (Iwahori et al., 1998; Ferris et al., 1993). The sequence of the ITS1 region of *A. fragariae* (Genbank Accession number AF119049) has been reported (Iwahori et al., 1998). Recently, "Universal" PCR primer sets have recently been used to obtain and identify the ITS1 and ITS2 sequence from *A. besseyi*, and *A. ritzemabosi* (Subbotin et al., unpublished). The potential to develop a molecular diagnostic assay for foliar nematode species may, thus, exist using the available ITS sequence of foliar nematode species to develop species-specific PCR primers. Once the primers are developed they must be validated for reliability of detection, specificity, and sensitivity (Hubschen et al., 2004). Optimally, foliar nematodes could be detected within an infected plant sample with sensitivity down to a single nematode.

Equally as important to an efficient diagnostic assay for foliar nematode infection of host plants is a representative and practical sampling strategy. An improved diagnostic assay can assist in development of a sampling protocol that could confidently detect nematodes before the infected plants are sold and transported across nursery, state, and national boundaries, even before the infected plants are showing symptoms. The majority of the phytonematode sampling protocols that have been developed are for root-pathogenic plant-parasitic nematodes. Root pathogenic nematodes are sampled for many reasons including to determine the spatial distribution of the nematodes throughout the field or soil column, for risk advisories based on population densities, management practices, and damage assessment (Abd-Elgawad and Hasabo, 1995; Duncan et al., 1994; Kashaija et al., 2004). For most of the root-pathogenic nematodes sampling protocols, soil cores are collected systematically throughout the field or sampling unit. Upon collection the nematodes are extracted from the soil cores using a nematode extraction technique such as a Baermann funnel or elutriation (Boag and Topham, 1984; Duncan, 1994; Kashaija et al, 2004; Singh and Sitaramaiah, 1994). However, the sampling protocols vary based on the crop, species, and biology of the nematode being sampled, parasitic relationship between host and the nematode, and the soil type (Singh and Sitaramaiah, 1994). Since foliar nematodes inhabit the foliage of the plant, it is necessary to sample the foliage. Currently, there is no systematic sampling protocol for the detection of foliar nematodes in ornamental plant nurseries.

Five fundamental concepts need to be considered to develop an accurate and sensitive plant sampling protocol for foliar nematode infestation. First is to develop an understanding of the nematode's basic biology. Second, a general knowledge of the distribution of the nematodes within the plant, which has not been extensively studied with foliar nematodes,

11

needs to be assessed. The third concept is sampling efficiency. Sampling protocols must be an efficient balance between economics and accuracy to a set degree of precision (Southwood and Henderson, 2000). The fourth concept is an understanding of the spread and spatial distribution of the foliar nematodes within the sampling area. Finally, a precise sampling size must be determined for the sampling plot (Southwood and Henderson, 2000). The spatial distribution and sample size can be determined by using one of several mathematical distributions, including Taylor's Power Law (Taylor, 1961). Taylor's Power Law uses the relationship between the population mean and variance to determine the dispersion patterns or the degree of aggregation of the nematodes within the sampling area. Taylor's Power Law has been effectively used to characterize the spatial distribution and develop a sampling size of several root pathogenic plant-parasitic nematodes (Abd-Elgawad and Hasabo, 1995; Boag and Topham, 1984; Kashaija et al.. 2004; Perry, 1983). Taylor's Power Law could, therefore, potentially provide an accurate assessment of the degree of aggregation for foliar nematodes within nursery blocks sampled. An algorithm derived from Taylor's Power Law, using the Taylor's Power Law coefficients, has been developed and found to provide an accurate sampling size for a designated plot (Wilson et al., 1983).

The focus of this thesis was to develop an efficient PCR technique for the detection of *Aphelenchoides fragariae*, a common pathogen in ornamental plant nurseries and greenhouses, that is more sensitive than current detection assays and requires limited nematology expertise. To effectively use such a PCR detection assay, a representative and practical sampling protocol for foliar nematodes must be developed for use in plant propagative stock, incoming, and existing plant stands. The coupling of improved detection

12

and sampling protocols for foliar nematodes can provide critical tools to reduce foliar

nematode spread and damage within ornamental plants.

LITERATURE CITED

Abd-Elgawad, M.M. and Hasabo, S.A. 1995. Spatial distribution of the phytonematode commonly in Egyptian erseem Clover (*Trifolium alexandrinum L.*) fields. *Fundamentals of Applied Nematology*. 18 (4): 329-334

Beckenbach, K., Blaxter, M., and Webster, J.M. 1999. Phylogeny of *Bursaphelenchus* species derived from analysis of ribosomal internal transcribed spacer DNA sequences. *Nematology*. 1(15): 539-548.

Boag, B. and Topham, P.B. 1984. Aggregation of Plant Parasitic Nematodes and Taylor's Power Law. *Nematologica*. 30:348-357.

Cherry, T., Szalanski, A.L., Todd, T.C., and Powers, T.O. 1997. The internal transcribed spacer region of *Belonolamius* (Nematoda: Belonolaimidae. *Journal of Nematology*. 29:23-29.

Chilton, N.B., Gasser, R.B., and Beveridge, I. 1995. Differences in a ribosomal DNA sequence of morphology indistinguishable species within the *Hypodontus macropi* complex (Nematoda: Strongyloidea). *International Journal for Parasitology*. 25: 647-651.

Christie, J.R. 1959. Plant nematodes, their bionomics and control. The H. and Drew Company, Jacksonville, Florida. 151-153.

Christie, J.R. 1932. Recent observations on the strawberry dwarf nematode in Massachusetts. *Plant Disease Reporter*. 16:113-114.

Coomans, A. 2002. Present status and future of nematode systematics. *Nematology*: 4: 573-582.

Decker, H., 1989. Leaf- Parasitic Nematodes. In *Plant Nematodes and Their Control*.
Sveshnikova, N. M. Ed. Kinderbrook, NY. E.J. Brill Publishing Company, 354-358.

De Maeseneer, J. 1964. Leaf- Browning of *Ficus* spp., new host plants of *Aphelenchoides fragariae* (Ritzema bos). *Nematologica*. 10:403-408.

Duncan, L.W., El-Morshedy, M.M. and McSorley, R. 1994. Sampling citrus fibrous roots of *Tylenchulus semipenetrans*. Journal of Nematology. 26(4): 442-451.

Fallas, G.A., Hahn, M.L., Fargette, M., Burrows, P.R., and Sarah, J.L. 1996. Molecular and biochemical diversity among isolates of *Radopholus* spp. from different areas of the world. *Journal of Nematology*. 28:422-430.

Fallas, G.A., Ferris, J.M., Faghihi, J. and Ireholm, A. 1994. Comparisons of isolates of *Heterodera avenae* using 2-D PAGE protein patterns and ribosomal DNA. *Journal of Nematology*. 26:144-151.

Ferris V.R., Ferris, J.M., and Faghihi, J. 1993. Variation in spacer ribosomal DNA in some cyst-forming species of plant-parasitic nematodes. *Fundamental and Applied Nematology*. 16:177-184.

Grewal, P.S. and Jagdale, G.B. 2001. Biology and management of foliar nematodes. *The Hosta Journal*. 32:64-66.

Harmey, J.H., and Harmey, M.A. 1993. Detection and Identification of *Bursaphelenchus* Species with DNA Fingerprinting and Polymerase Chain Reaction. *Journal of Nematology*. 25(3): 406-415.

Hubschen, J., Kling, L., Ulrike, I., Zinkernagel, V., Bosselut, N., Esmenjaud, D., Brown, D.J.F., and Neilson, R. 2004. Validation of the specificity and sensitivity of species-specific primers that provide a reliable molecular diagnostic *for Xiphinema*

15

diversicaudatum, X. index, and *X. vuittenezi. European Journal of Plant Pathology.*
110:779-788.

Hunt, D.J. 1993. Aphelenchida, Longidoridae, and Trichodoridae: Their Systematics and
Bionomics. CAB International, University Press, Cambridge. 57-65.

Hunter, J.E., Ko, W.H., Kunimoto, R.K., and Higaki, T. 1974. A foliar disease of Anthurium
seedlings caused by *Aphelenchoides fragariae. Phytopathology.* 64: 267-268.

Ibrahim, S.K., Perry, R.N., Burrows, P.R., and Hooper, D.J. 1994. Differentiation of Species
and Population of *Aphelenchoides* and of *Ditylenchus angustus* Using a Fragment of
Ribosomal DNA. *Journal of Nematology.* 26(4): 412-421.

Iwahori, H., Tsuda, K., Kanzaki, N., Izui, K., and Futai, K. 1998. PCR-RFLP and sequencing
analysis of ribosomal DNA of *Bursaphelenchus* nematodes related to pine wilt
disease. *Fundamentals of Applied Nematology.* 21(6): 655-666.

Jagdale, G.B., and Grewal, P.S. 2002. Identification of alternatives for the management of
foliar nematodes in floriculture. *Pest Management Science.* 58: 451-458.

Jagdale, G.B., and Grewal, P.S. 2004. Effectiveness of a Hot Water Drench for the Control
of Foliar Nematodes Aphelenchoides fragariae in Floriculture. *Journal of
Nemataology.* 36(1): 49-53.

Jagdale, G.B. and Grewal, P.S. 2006. Infection Behavior and Overwintering Survival of
Foliar Nematodes *Aphelenchoides fragariae*, on Hosta. *Journal of Nematology.* 38(1):
130-136.

Jenkins, W.R., and Taylor, D.P. 1967. Plant Nematology. Reinhold Publishing Corp., New
York. 163-172.

Johnson, A.W. and Gill, D.L. 1975. Chemical control of foliar nematodes (*Aphelenchoides fragariae*) on 'Fluffy Ruffles' fern. *Plant Disease Reporter*. 59(9) 772-774.

Kashaija, I.N., McIntyre, B.D., Ssali, H. and Kizito, F. 2004. Spatial distribution of roots, nematode populations and root necrosis in highland banana in Uganda. Nematology. 6(1): 7-12.

Klinkenberg, C.H. 1955. Nematode diseases of Strawberry in the Netherlands. *Plant Disease Reporter*. 39:603-606.

Klinger, J. 1970. The Reaction of *Aphelenchoides fragariae* to slit-like micro-openings and to stomata diffusion gases. *Nematologica*. 16: 417-422.

La Mondia, J.A., 1999. Efficacy of Insecticides for Control of *Aphelenchoides fragariae* and *Ditylenchus dipsaci* in Flowering Perennial Ornamentals. *Journal of Nematology*. 31(4S): 644-649.

Mac Lachlan, J.B. and Duggan, J.J. 1979. An effective method for hot-water treatment and propagation of strawberry runners. Irish Journal of Agriculture Research 18:301-304.

Maggenti, A. 1981. Aphelenchoid Parasites of Plant Shoots: Bud and Foliar Nematodes. In *General Nematology*. Starr, M.P. Ed. New York, NY USA: Springer-Verlag, 209-211.

Mor, M. and Spiegel, Y. 1993. *Ruscus hypophyllum*: A New Host for *Aphelenchoides fragariae*. *Journal of Nematology*. 25(2):312-313.

Nasmith, C.G., Speranzini, D., Jeng, R., and Hubbes, M. 1996. RFLP analysis of PCR amplified ITS and 26Ss ribosomal genes of selected entomopathogenic nematodes (Steinernematidae, Heterorhibditidae). *Journal of Nematology*. 28:15-25.

Noel, G.R. 1994. Hosta: A New Host Record for *Aphelenchoides fragariae*. *Plant Disease*. 78: 924.

Oliveira, C.M., Fenton, B., Malloch, G., Brown, D.J.F., and Neilson, R. 2005. Development of species-specific primers for the ectoparasitic nematode species *Xiphinema brevicolle, X. diffusum, X. elongatum, X. ifacolum,* and *X. longicaudatum* (Nematoda: Longidoridae) based on ribosomal DNA sequences. *Annuals of Applied Biology*. 146: 281-288.

Plakidas, A.G. 1928. Strawberry Dwarf. *Phytopathology*. 18(5)439-444.

Perry, J.N. 1983. Effects of spatial heterogeneity of Jones's Model of Cyst Nematode Population Dynamics and Crop Root Damage. Journal of Applied Ecology. 20: 849-856.

Powers, T.O., and Harris, T.S. 1993. A Polymerase Chain Reaction Method for Identification of Five Major *Meloidogyne* Species. *Journal of Nematology*. 25(1): 1-6.

Powers, T.O., Todd. T.C., Burn ell, A.M., Murray, P.C.B., Fleming, C.C., Szalanski, A.L., Adams, B.A., and Harris, T.S., 1997. The renal Internal Transcribed Spacer Region as a Taxonomic Marker for Nematodes. *Journal of Nematology*. 24(4): 441-450.

Qui, J., Westerdahl, B.B., Buchner, R.P., and Anderson, C.A. 1993. Refinement of hot water treatment for management of *Aphelenchoides fragariae* in Strawberry. Supplement to the *Journal of Nematology*. 25:795-799.

Riedel, R.M. and Powell, C.C. 1974. Control of *Aphelenchoides fragariae* on *Rieger Begonia* with Oxamyl. *Plant Disease Reporter*. 58 (10): 911-913.

Sanwal, K. C. 1961 A key to the species of the nematode genus *Aphelenchoides* Fischer, 1894. *Canadian Journal of Zoology*. 39:143-148.

Sanwal, K.C. 1959. A simple method of rearing pure populations of the foliar nematode,

    Aphelenchoides ritzemabosi, in the laboratory. *Canadian Journal of Zoology*. 37:707-

    711.

Schulze, L. 2003. Federally registered restricted use pesticides. Clemson University Pesticide

    information Program. 1-16, http://entweb.clemson.edu/pesticid

    /document/fedrup.htm.

Singh, R. S. and Sitaramaiah, K. 1994. Plant Pathogens: The Plant Parasitic Nematodes.

    International Science Publisher, New York, NY. 261-289.

Shields, R., Fleming, C.C., and Stratford, R. 1996. Identification of potato cyst nematodes

    using the polymerase chain reaction. *Fundamental and Applied Nematology*. 19:167-

    173.

Siddiqi, M.R. 1975. *Aphelenchoides fragariae*. CIH Descriptions of plant parasitic

    nematodes. CAB International, Wallingford, GB. 5(74):4

Siddiqi, M.R. 1974. *Aphelenchoides ritzemabosi*. CIH Descriptions of plant-parasitic

    nematodes. St Albans, Herts, UK. Commonwealth Institute of Helminthology,

    3(32):4.

Southwood, T.R.E. and Henderson, P.A. 2000. Ecological methods Third Edition. Blackwell

    Science Led. Malden, MA. 15-26.

Stevenson, L.A., Chilton, N.B., and Gasser, R.B., 1995. Differentiation of *Haemonchus*

    placei from *H. contortus* (Nematoda: Trichostrongylidae) by the ribosomal DNA

    second internal transcribed spacer. *International Journal of Parasitology*. 25:483-

    488.

Stewart, F.H. 1921. The anatomy and Biology of the parasitic Aphelenchi. *Parasitology*. 13: 160.

Stiener, G. 1932. The successful transfer of *Aphelenchoides ritzema-bosi* from chrysanthemum to strawberry plants. *Journal of Parasitology*. 19(1): 90.

Subbotin, S.A., Waeyenberge, L., and Moens, M. 2000. Identification of cyst forming nematodes of the genus *Heterodera* (Nematoda: Heteroderidae) based on the ribosomal DNA-RFLP. *Nematology*. 2:153-164.

Subbotin, S.A., Waeyenberge, L., Malokanova, I.A., and Moens, M. 1999. Identification of *Heterodera avenae* group species by morphometrics and rDNA-RFLPs. *Nematology*. 1: 195-207.

Szalanski, A.L., Sui, D.D., Harris, T.S., and Powers, T.O. 1997, Identification of cyst nematodes of agronomic and regulatory concern by PCR-RFLP of ITS1. *Journal of Nematology*. 29:255-267.

Taylor, L. R. 1961. Aggregation, Variance, and the Mean. Nature. 189: 732-735.

Thiery, M., and Mugniery. 1996. Interspecific rDNA restriction fragment length polymorphism in *Globodera* species, parasites of solanaceous. *Fundamental and Applied Nematology*. 19: 471-479.

Thorne, G. 1961. Principles of Nematology. McGraw- Hill Book Company, Inc. New York, NY. 410-427.

Todd, E.H., and Atkins, J.G., 1958. White Tip Disease of Rice. I. Symptoms, Laboratory Culture of Nematodes, and Pathogenicity Tests. *Phytopathology*. 48: 632-637.

Uehara, T., Mizukubo, T., Kushida, A., and Momota, Y. 1998. Identification of *Pratylenchus coffeae* and *P. loosi* using specific primers for PCR amplification of ribosomal DNA. *Nematologica*. 44:357-368.

USDA. 1978. Strawberry Diseases (Revised). Farmers' Bull. No. 2140.

Vrain, T.C. and McNamara, D.G. 1994 Potential for identification of quarantine nematodes by PCR. EPPO Bulletin 24: 453-458.

Vrain, T.C., Wakarchuk, D.A., Levesque, A.C., and Hamilton, R.I. 1992. Intraspecific rDNA restriction fragment length polymorphism in the *Xiphinema americanum* group. *Fundamental and Applied Nematology*. 15:563-573.

Vovlas, N., Minuto, A., Garibaldi, A., Troccoli, A., and Lamberti, F. 2005. Identification and histopathology of the foliar nematode *Aphelenchoides ritzemabosi* (Nematoda: Aphelenchoididae) on basil in Italy. *Nematology*. 7(2): 301-308.

Wang, X., Bosselut, N., Castagnone, C., Voisin, R., Abad, P., and Esmenjaud, D. 2003. Multiplex polymerase chain reaction identification of single individuals of the Longidorid nematodes *Xiphinema index, X. diversicaudatum, X. vuittenezi,* and *X. italiae* using species specific primers from ribosomal genes. *Phytopathology*. 93: 160-166.

Wallace, H.R. 1961. Browning of Chrysanthemum leaves infested with *Aphelenchoides ritzemabosi*. *Nematologica*. 6,.

Wallace, H.R. 1959. Movement of eelworms V. Observations of *Aphelenchoides ritzemabosi* on florist chrysanthemum. *Annuals of Applied Biology*. 47: 350-360

Wendt, K.R., Swart, A., Varian, T.C., and Webster, J.M. 1995. *Ditylenchus africanus* sp. n.

from South Africa—a morphological and molecular characterization. *Fundamental

and Applied Nematology*. 18:241-250.

Wilson, L.T., Gonzalez, D., Leigh, T.F., Maggi, V., Foristiere, C., and Goodell, P. 1983.

Within-Plant Distribution of Spider Mites (Acari: Tetranychidae) on Cotton: A

Developing Implementable Monitoring Program. Environmental Entomology. 12:

128-134.

Whitehead, A.G., and Hemming, J.R., 1965. A comparison of some quantitative methods of

extracting small vermiform nematodes from soil. *Annals of Applied Biology*. 55:25-

38.

Yamanda, E. and Takakura, S. 1989. Effect of chemical and hot water treatment for control

of the strawberry nematode *Aphelenchoides fragariae* on nematode-infested lily

bulbs. *Jap. Journal of Nematology*. 18:15-21.

Yokoo, T. 1948. Taxonomy of *Aphelenchoides oryzae* Yokoo n. sp., a nematode parasite of

rice, Ann. Phytopathology Society of Japan. 13:40-43.

Zijlstra, C., Lever, A.E.M., Uenk, B.C., and Van Silfhout, C.H. 1995. Differences between

ITS regions of isolates of root-knot nematodes *Meloidogyne hapla* and *M. chitwoodi*.

Phytopathology. 85:1231-1237.

Zijljstra, C., Uenk, B.J., and Van Silfhout, C.H. 1997. A reliable, precise method to

differentiate species of root-knot nematodes in mixtures on the basis of ITS-RFLPs.

Fundamental and Applied Nematology. 20:59-63.

CHAPTER 1. DEVELOPMENT OF A MOLECULAR DETECTION ASSAY SPECIFIC FOR *APHELENCHOIDES FRAGARIAE*

ABSTRACT

The objective of this study was to develop a PCR technique for the detection of *Aphelenchoides fragariae*, a common pathogen in nurseries and greenhouses. The standard test currently used to detect the nematode relies on the ability of the nematode to crawl or swim out of a leaf submerged in water, as well as the ability of a person to recognize specific small morphological characteristics. This method is both time consuming and often not reliable because of contamination from other saprophytic nematodes and the minute differences in characteristics of other non-pathogenic *Aphelenchoides* species. In order to replace the current standard method, the PCR detection method needs to be specific, rapid, and adaptable to routine analysis.

The ITS1 sequence for *A. fragariae* were used to develop sequence-specific primers that were specific to only *A. fragariae*. The sequences of seven A. fragariae, isolated from different geographical areas throughout North Carolina, showed that within the area amplified by the species-specific primers developed was 100% homologous to the sequence that the primers were developed from as well as from a known culture of *A. fragariae* (Figure 3). The ITS1 region of a recent comparison with sequences recently added to the GenBank database since the time this study was originally initiated, show 80% sequence homology within the amplified region of the ITS1 region with A. fragariae in the Netherlands. (EF213107, Pham et al., 2007) From this information the ITS1 region appears to be mainly conserved throughout this region. Specimens of *Aphelenchoides* spp. were recovered from 121 plant samples. Based on morphology, 89 of these isolates were identified as *A. fragariae,*

the nematodes from the remaining 32 plant samples either were not identified or did not

swim out of the plant tissue but was detected using the PCR-assay. Genomic DNA

containing both plant and nematode DNA produced a 169-bp fragment when PCR-amplified

with primers FragF1 and FragR1 for 100 of the isolates identified as *A. fragariae* (Table 1).

By using several isolates collected in NC, we demonstrated the utility of this newly

developed primer pair for discriminating *A. fragariae*.

INTRODUCTION

The genus *Aphelenchoides* contains approximately 143 species of nematodes (Hunt, 1993). Many of these *Aphelenchoides* species are saprophytic in the soil and water, plant-parasitic, and/or associated with some insects (Sanwal, 1961). Among the plant pathogens, three *Aphelenchoides* species are important in the greenhouse or nursery industry due to the host range; *Aphelenchoides fragariae*, *Aphelenchoides besseyi*, and *Aphelenchoides ritzemabosi*. Foliar nematodes are believed to feed on the mesophyll and parenchyma tissues of leaves or fronds (Decker, 1989, Maggenti, 1981). The symptoms appear as vein delineated lesions due to the inability of the nematode to cross the major leaf veins once inside the plant. (Maggenti, 1981). Each *A. fragariae* generation takes 10 to 11 days to reach sexual maturity under optimum temperatures of 18ºC (Decker, 1989). If the conditions remain favorable, many generations may rapidly increase, resulting in a heavy infection per leaf (Decker, 1989).

Aphelenchoides fragariae infects over 250 species of ornamental plants, including a large number of herbaceous and woody perennials. The total wholesale value of herbaceous perennial plants grown by U.S. operators with individual sales greater than $100,000 was $704 million in 2005, up 3% from 2004 (USDA, 2006). In North Carolina, 28.3 million pots of herbaceous perennials were sold in 2005, making North Carolina the eighth largest producer of herbaceous perennials among the 36 states surveyed (USDA, 2006). Propagated plant material, which includes herbaceous perennials, accounted for an additional $439 million in sales in 2005 with the largest volume of material produced in Florida (USDA, 2006). Most plant species do not exhibit symptoms of foliar nematode damage until the plant is heavily infected with nematodes. Development of symptoms may take from weeks-to-

months depending upon the host plant and environmental conditions in the nursery or greenhouse, making early detection and eradication problematic.

The management of foliar nematodes is very difficult due to the lack of control methods. Currently, there is only one pesticide, a miticide, labeled for indoor use on ornamental crops for the control of foliar nematodes. However, while this product will reduce the foliar nematode population, it does not eradicate them. A hot water drench has been developed and tested on hosta (*Hosta* sp) and ferns (*Matteuccia pensulvanica*) with some success (Jagdale and Grewal, 2004). However, using hot water is not a method that can be used on a widespread basis in large commercial nurseries or greenhouses (Jagdale and Grewal, 2004). Furthermore, many ornamental plants are sensitive to the excessive temperature and length of time of the hot water drench necessary to kill foliar nematodes (Jagdale and Grewal, 2004). Currently, the most efficacious control strategy is exclusion and eradication, including the use nematode-free propagative-stock plants. There is concern among growers that foliar nematode-infected plants and cut foliage are being sold and transported across nursery, state, and national boundaries and subsequently infesting previously non-infested plant nurseries and propagation facilities (Jagdale and Grewal, 2006). The current water extraction of foliar nematodes from plant samples requires several days of incubation per sample and the expertise of a trained nematologist for positive identification of nematode species (Jagdale and Grewal, 2002). The increased incidence of foliar nematode infestation of ornamental plant nurseries and limited management options has necessitated the need to develop an accurate, sensitive, and practical method to screen plants for foliar nematode infection prior to disease symptom development.

The ribosomal DNA (rDNA) of plant-parasitic nematodes consists of three individual subunits (18S, 5.8S, and 28S) with two internal transcribed spacer (ITS1 and ITS2) regions (Powers et al., 1997). Selection for functional conservation in the rRNA-coding 18S, 5.8S, and 28S subunits imposes limited subunit rDNA sequence variability across species as compared to the more extensive variability in the non-coding ITS rDNA sequences that are relatively free of selection pressure (Powers et al., 1997). The variability of rDNA sequence within the ITS regions can be used for differentiation of nematode species (Iwahori et al., 1998). Nematologists have used the rDNA ITS regions to identify plant-parasitic species, animal-parasitic species, and insect-parasitic species of nematodes (Beckenbach et al., 1999; Cherry et al., 1997; Chilton et al., 1995; Fallas et al., 1996; 1993; Harmey and Harmey, 1993; Ibraham et al., 1994; Joyce et al., 1994; Nasmith et al., 1996; Powers and Harris, 1993; Powers et al., 1997; Stevenson et al., 1995; Szalanski et al., 1997; Thiery and Mugniery, 1996; Vrain and McNamara, 1994; Vrain et al., 1992; Wendt et al., 1995; Zijlstra et al. 1997, 1995). "Universal" primer sets for polymerase chain reaction (PCR) have also been developed to amplify previously unknown rDNA sequence and obtain the ITS1 and ITS2 regions (Iwahori et al., 1998). The ability to amplify the rDNA ITS region from individual nematodes provides potential for powerful analyses of nematode population structure or to develop sensitive diagnostic assays (Powers et al., 1997). The use of *A. fragariae* as a standard for PCR-RFLP analyses (Iwahori et al., 1998) provided a partial rDNA ITS1 sequence (GenBank Accession: AF119049) that was used here as a starting point to develop a sensitive and species-specific assay to detect infection of plant hosts by *A. fragariae*. The resulting assay can identify individual *A. fragariae* within both symptomatic and asymptomatic host plant tissue.

MATERIALS AND METHODS

Morphological identification of *Aphelenchoide*s species

Foliar nematodes extracted from naturally-infected plant tissues were identified using a compound light microscope with differential interference contrast imaging to observe characteristics unique to each species. At least three adult females from each sample were used to help ensure more accurate species identification. A large, round median bulb (metacorpus) is a distinctive feature of all nematodes belonging to the Order *Aphelenchida*. In addition to the large metacorpus, each nematode was examined for three species-specific characteristics as referenced by Hunt (1993) using 1000× magnification. For *Aphelenchoides fragariae*, the head (cephalic) region appears high and almost continuous with the body, the tail ends in a blunt point, and the oocytes are in a single-file line (Figure 1). For *Aphelenchoides besseyi,* the cephalic region appears rounded and slightly wider than the body, the tail ends in a three-to-four pointed terminus (mucro), and the oocytes are in two to four rows. For *Aphelenchoides ritzemabosi*, the cephalic region is hemispherical and slightly wider than the body, the tail ends with two-to-four mucro, and oocytes are in multiple rows.

DNA extraction from nematodes

A. fragariae propagated on alfalfa plants grown in agar-based monoxenic culture on Gamborgs B-5 media (Sigma-Aldrich, St. Louis, MO) in 100×15-mm plastic Petri dishes were used as a known source of DNA. The cultures were maintained in a plant growth chamber at 26°C with a 12-hr day and 12-hr night. A minimum of 23 days after subculture, the entire plant-nematode-agar culture was broken apart using a metal spatula, inverted onto a Kimwipe-lined mesh screen suspended above a shallow bowl of water, and covered with foil for 48 hr to allow the nematodes to emerge from the tissue and into the water. The

extraction suspension was centrifuged in a 1.5-ml microcentrifuge tube at 8,000 rpm for 1

minute to pellet the nematodes. The supernatant was removed and the process was repeated

until all of the extraction water was collected, spun, and removed. The nematode pellet,

composed of mixed stages of juvenile and adults, was transferred to a 2.0 ml conical

microtube filled halfway with 1-mm glass beads and 180 µl of lysis Buffer ATL (Qiagen,

Inc., Valencia, CA.). Nematodes were subsequently disrupted in a Mini-BeadBeater-1

(Biospec Products, Inc., Bartlesville, OK) for 10 seconds at 2500 rpm and total genomic

DNA was extracted using a DNeasy Blood and Tissue kit (Qiagen, Inc., Valencia, CA)

following the Animal Tissues Spin Protocol supplied by the manufacturer. The DNA was

eluted in 50 µl of kit elution buffer and stored at -20°C. Nucleic acids were not quantified

prior to use in PCR amplifications.

PCR primer design for *A. fragariae*

Universal primers 18S(ITSFWD) and 5.8S(REV) obtained from Byron Adams,

Brigham Young University (unpublished) were originally used to amplify the partial

sequence of the 18S rRNA gene, the full sequence of the ITS1 region, and a partial sequence

of the 5.8S rRNA gene from template DNA extracted from cultured *A fragariae*. PCR

amplifications were performed in 50-µl reactions containing 3.0 µl of total DNA, 1.5 units of

Platinum *Taq* DNA Polymerase (Invitrogen, Inc.,Carlsbad, CA), 1× PCR buffer (20 mM

Tris-HCL (pH 8.4), 50 mM KCl), 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.2 µM of each

oligonucleotide primer, and sterile molecular-grade water to volume. PCR products were

separated on a 1.5% agarose gel with 1× TBE. The resulting 450-bp fragment was cut from

the gel and purified using a MiniElute Gel Extraction Kit (Qiagen, Inc., Valencia, CA). Two

microliters of purified DNA was used for electrophoresis to ensure DNA was present.

Purified DNA was sent to The DNA Analysis Facility of the Iowa State University Office of

Biotechnology for sequencing. DNA sequence data were analyzed using Vector NTI®

Advance 10.3.0 (Invitrogen, Inc., Carlsbad, CA).

DNA sequence of the ITS1 region from cultured *A. fragariae* obtained with universal

primers was aligned with the reported ITS1 region (GenBank Accession: AF119049) of *A.*

fragariae (Iwahori et al., 1998) and also compared with reported ITS sequences in GenBank

to determine potential similarity among other nematodes, insects, plants, or plant pathogens

using the Basic Local Alignment Search Tool (BLASTn). From the combined analyses,

forward primer AFragF1, 5'-GCAAGTGCTATGCGATCTTCT-3' (38-to-58 bp) and reverse

primer AFragR1, 5'-GCCACATCGGGTCATTATTT-3' (187-to-206 bp) were designed

using Primer3 (Rozen and Skaletsky, 2000) from unique sequences near the ends of the

aligned *A. fragariae* ITS1 regions. The AFragF1 and AFragR1 primers were rated for

amplification success based on potential secondary structural problems including hairpins,

dimers, and palidromes using Net Primer (PREMIER Biosoft International, Palo Alto, CA)

and predicted to amplify a PCR product 169-bp in length.

PCR amplification

Optimized and reproducible PCR amplifications using primers AFragF1 and

AFragR1 were performed in 25-µl reactions containing 2.0 µl of total DNA, 1.25 units of

Platinum *Taq* DNA Polymerase (Invitrogen Corp., Carlsbad, CA), 1× PCR buffer (20 mM

Tris-HCL (pH 8.4), 50 mM KCl), 1 mM $MgCl_2$, 200 µM of each dNTP, 0.4 µM of each

oligonucleotide primer, and sterile molecular-grade water to volume. A reaction without

DNA template was always included as a negative control. Reactions were carried out in a

GeneAmp PCR System 2700 thermal cycler (Applied Biosystems, Foster City, CA)

programmed according to the following: an initial denaturation step at 94ºC for 2 min followed by 40 cycles each consisting of denaturation at 94ºC for 1 min, annealing at 53ºC for 40 s, and extension at 72ºC for 1 min, with a single final extension at 72ºC for 10 min. The thermal cycler was set to hold at 4ºC after the program was complete. Ten microliter aliquots of the PCR products were added to 2 µl of 6× dye marker solution (0.25% bromophenol blue, 30% glycerol) and separated on a 1.5% agarose gel containing 0.15 µg/ml ethidium bromide buffered in 1× TBE run at 100 V for 1 hour, and visualized under UV light.

DNA Sequencing of the ITS1 region for comparison of *A. fragariae* isolates

To detect variability in the ITS1 region among *A. fragariae* isolates, universal primers 18S(ITSFWD) and 5.8S(REV) obtained from Byron Adams, Brigham Young University (unpublished) were used to amplify the partial sequence of the 18S rRNA gene, the full sequence of the ITS1 region, and a partial sequence of the 5.8S rRNA gene from DNA of seven nematode populations extracted from naturally-infected plants collected in North Carolina. PCR amplifications were performed in 50-µl reactions containing 3.0 µl of total DNA, 1.5 units of Platinum *Taq* DNA Polymerase (Invitrogen, Inc., Carlsbad, CA), 1× PCR buffer (20 mM Tris-HCL (pH 8.4), 50 mM KCl), 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.2 µM of each oligonucleotide primer, and sterile molecular-grade water to volume. PCR products were separated on a 1.5% agarose gel with 1× TBE. The resulting 450-bp fragment was cut from the gel and purified using a MinElute Gel Extraction Kit (Qiagen, Inc., Valencia, CA). Two microliters of purified DNA was used for electrophoresis to ensure DNA was present. Purified DNA was sent to The DNA Analysis Facility of the Iowa State University Office of Biotechnology for sequencing. Samples were run on the facility's

Applied Biosystems Inc. ABI 3730x1 DNA Analyzer. Data were analyzed using Vector

NTI® Advance 10.3.0 (Invitrogen, Inc., Valencia, CA). Sequences were aligned to check for

mismatches, ambiguity and insertion/deletion events in each ITS1 region.

Specificity of the *A. fragariae*-specific PCR assay

DNA extracted from seven independent isolates morphologically identified as *A.

fragariae* was used as PCR template with the universal primers 18S(ITSFWD) and

5.8S(REV) from Byron Adams, Brigham Young University as described above. Sequence of

the full rDNA ITS1 region and the predicted 169 ITS1 fragment obtained with primers

AFragF1/AFragR1 were aligned and compared among the seven A. *fragariae* isolates. The

sequence of the consensus 169bp *A. fragariae* ITS1 fragment was also aligned and compared

with ITS1 sequence of *A. besseyi* and *A. ritzemabosi* provided by Sergei Subbotin of

Univeristy of California, Riverside (Chizhov, et al., 2006)

The primer specificity of AFragF1/AFragR1 were also analyzed in PCR reactions

using template DNA extracted from several cultured nematode species including

Meloidogyne incognita, *Heterodera schachtii*, *Pratylenchus penetrans*, *Caenorhabditis

elegans*, and *Ditylenchus dipsaci, Aphelenchoides besseyi* (from Sergei Subbotin, UCR) , and

a DMSO-fixed, unidentified species of Aphelenchoides (from Luis Gomez at the University

of Costa Rica). Primer specificity of AFragF1/AFragR1 was also evaluated against DNA

extracted from common nursery and greenhouse pests and pathogens including obscure

mealybug (*Pseudococcus affinis*), whiteflies (*Trialeurodes* spp.), and spider mites

(*Tetranychus* sp.) collected from North Carolina State University's greenhouse facility, and

aphids (*Microsiphum* spp. and *Myzus persicae*) collected in a landscape perennial bed in

Raleigh, NC. DNA from these pathogens was extracted using the Qiagen DNeasy Blood and

Tissue Kit (Valencia, CA) according to the manufacturer's Animal Tissues protocol. The

DNA was eluted in two steps using 50 µl of the elution buffer supplied by the manufacturer,

and stored at -20°C. Cheryle O'Donnell at the University of California, Davis kindly

provided DNA of Western flower thrips (*Frankliniella occidentalis*). DNA was also

extracted from mycelia of the fungal pathogen *Botrytis cinerea*, grown overnight in spore-

inoculated V8 broth and extracted using a PUREGENE® DNA Purification Kit for Yeast and

Gram-positive Bacteria (Gentra Systems, Minneapolis, MN). DNA from pure cultures of

bacteria *Xanthomonas campestris* pv. *zinniae* and a *Pseudomonas* sp. (isolated from Lantana)

was extracted and purified using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA)

and evaluated for cross-reactivity with primers FragF1 and FragR1. Extracted DNA from

several, healthy host plants including *Lantana* 'New Gold', *Salvia* microphylla, *Verbena*

'Snowflurry', *Heuchera* 'Frosted Violet', *Dahlia* 'Bishop of Canterbury' and *Asplenium*

nidus was also used as template in the PCR-reaction mixture to determine if the *A. fragariae*-

specific primers had any cross-reactivity to plant rDNA.

Sensitivity of the *A. fragariae*-specific PCR assay

DNA was extracted from decreasing numbers of nematodes collected from a

monoxenic culture of *A. fragariae*. In the first assay, nematodes were hand-picked from the

extraction suspension and pooled in groups of 50, 20, 15, 10, 5, 4, 3, 2, and 1 individual

nematode for DNA extraction using the Qiagen DNeasy Blood and Tissue kit (Valencia,

CA). For the second assay, DNA from a single *A. fragariae* nematode was extracted together

with increasing numbers of 6-mm leaf disks (from 1 to 20 disks) collected from uninfected

Asplenium nidus leaf tissue. The leaf disks were weighed to determine when the maximum

amount of plant tissue (100 mg) recommended by the manufacturer was reached, to avoid a

potential reduction in DNA yield and purity. This second assay was repeated two more times using leaf disks removed from uninfected *Heuchera* 'Frosted Violet,' or *Dahlia* 'Bishop of Canterbury' leaves. DNA extracted from leaf disks, without the addition of a nematode, was used as a negative control. The third assay varied the number of individual *A. fragariae* nematodes collected from the monoxenic cultures, but kept the number of leaf disks constant. Increasing increments of nematodes from 1 to 1,000 were hand-picked and combined with three non-infected *A. nidus* leaf disks, with total DNA extracted using the Qiagen DNeasy Plant Mini Kit. Extracted DNA from each sensitivity assay was used as template in PCR-reaction mixtures with the *A. fragariae*-specific primer set, AFragF1 and AFragR1.

Detection of foliar nematodes in infected plant specimens

Herbaceous perennial and woody ornamental plants exhibiting foliar nematode symptoms were collected at wholesale and retail nurseries, retail garden centers, and outdoor landscapes in North Carolina from 2004 through 2007 (Table 1). Symptomatic leaf tissue from each plant sample was assayed for the presence of foliar nematodes using both water extraction (Jagdale and Grewal, 2002) and PCR-amplification of the total plant/nematode DNA using primers AFragF1 and AFragR1. One symptomatic leaf per plant was divided in half. One half was thinly sliced with scissors and placed in a 60×15-mm Petri dish containing just enough water to cover the tissue for water extraction. Extraction dishes were incubated at room temperature for 24 to 48 hr, and examined under a dissecting microscope to confirm the presence of nematodes. Nematodes in the collection dishes were pelleted as described above and fixed with 10% formaldehyde for later identification to species using morphology under light microscopy. A maximum 1.0 mg of leaf tissue was removed from the second half of the symptomatic leaf and placed in a 2-ml conical tube containing two 5-

mm glass beads. The tissue was frozen in liquid nitrogen by immersing the tube for 30

seconds and subsequently disrupted in a Mini-BeadBeater-1 (Biospec Products, Inc.,

Bartletsville, OK) for 10 seconds at 2500 rpm. This process was repeated three times.

Ground tissue was processed for DNA extraction by use of a DNeasy Plant Mini Kit (Qiagen,

Inc., Valencia, CA) following the manufacturer's protocol. Total genomic DNA was eluted

in a final volume of 100 µl and stored at -20°C. Extracted DNA from each assay was used as

template in PCR-reaction mixtures with the *A. fragariae*-specific primer set, AFragF1 and

AFragR1.

In a separate experiment, DNA extracted from three unidentified *Aphelenchoides*

species (isolated from naturally infected *Cheilanthes wrightii*, *Chrysanthemum* 'Color Echo,'

and *Dahlia* 'Bishop of Canterbury' plants) that deviated in one or more morphological

characteristics typical for *A. fragariae* was used as template for AFragF1/AFragR1 PCR.

Any resulting PCR fragment visualized on a 1.5% agarose electrophoretic gel was excised

using a MinElute Gel Extraction Kit (Qiagen, Inc., Valencia, CA) according to the

manufacturer's gel extraction spin protocol. The purified product was ligated into pCR$^®$2.1

and transformed into chemically competent *Escherichia coli* One Shot$^®$ Top10F′ cells using

the One Shot$^®$ Chemical Transformation Protocol included in the TA Cloning$^®$ Kit

(Invitrogen Corp., Carlsbad, CA). Successful transformants were selected and incubated

overnight in LB (Luria-Bertani) broth containing 50-µg/ml ampicillin. Plasmid DNA was

extracted from the *E. coli* cultures using an eppendorf FastPlasmid™ Mini kit (Brinkman

Instruments, Inc., Westbury, NY) according to the manufacturer's protocol. Purified plasmid

DNA was cut using the restriction enzyme *EcoRI*, and subsequently visualized on a 1.5%

agarose gel to confirm the presence any PCR product insert. Corresponding plasmid

constructs from PCR-positive clones were submitted for sequencing to the University of
Florida DNA Sequencing Core Laboratory at the Interdisciplinary Center for Biotechnology
Research (ICBR) in Gainesville, Florida. Sequences generated from the three nematode
populations were analyzed using the b12seq algorithm as implemented in BLASTn, and
aligned with the consensus A. *fragariae* ITS1 sequence.

NaOH extraction of *A. fragariae* template DNA

Experiments were conducted to test NaOH lysis as an alternative method to DNeasy
kits for rapid extraction of template DNA for AFragF1/AFragR1 PCR. Nine healthy
(nonsymptomatic) and nine nematode-infested, 6-mm leaf disks were removed from several
plant samples. Three leaf disks from each set, healthy vs. infected, were i) extracted in water
to confirm the presence of nematodes and used for DNA extraction using both a DNeasy
Plant Mini Kit (Qiagen, Inc., Valencia, CA), and NaOH lysis. For the NaOH extraction
method, three leaf disks were ground in 150 μl of 0.5N NaOH using a polypropylene Pellet
Pestle[©] (Kimble/Kontes, Vineland, New Jersey), after which 5 μl was transferred
immediately into a sterile 1.5 ml microcentrifuge tube containing 495 μl of 100 mM Tris-
HCl, pH 8.0 (Wang et al., 1993). Serial dilutions were prepared using 100 mM Tris-HCL, pH
8.3, and 2 or 3 μl of the dilute suspensions were added to 25 μl of PCR master mix.
Extracted DNA from each assay was used as template in PCR-reaction mixtures with the *A.*
fragariae-specific primer set, AFragF1 and AFragR1.

RESULTS

A. fragariae **Primer Design and ITS1 Sequence Alignments**

Divergence in 3' DNA sequence between aligned ITS1 sequences of *A. fragariae*, *A. besseyi*, and *A. ritzemabosi* (Figure 2) was detected among full ITS1 regions generated with the universal primers 18S(ITSFWD) and 5.8S(REV). DNA sequence of the internal 169-bp ITS1 fragment generated with primers AfragF1 and AfragR1, however, was 100% identical among seven independent isolates of *A. fragariae* (confirmed morphologically) and the reported sequence (GenBank Accession No. AF119049) of Iwahori et al. (1998).

Specificity of the *A. fragariae*-specific PCR assay

The AfragF1/AfragR1 primer pair reproducibly directed amplification of the rDNA-ITS1 region of *A. fragariae,* generating products 169-bp in length using total genomic DNA extracted from individual nematodes, populations of nematodes, and leaf tissue samples naturally-infected with *A. fragariae* (Figures 3-7). PCR-reaction mixtures containing primers AFragF1 and AFragR1 did not produce an amplification product using extracted DNA from the nematodes species *Meloidogyne incognita*, *Heterodera schachtii*, *Pratylenchus penetrans*, *Caenorhabditis elegans*, *Ditylenchus dipsaci*, *Aphelenchoides besseyi*, or using DNA from the unidentified *Aphelenchoides* sp. collected in Costa Rica. (Figure 3)

Primers AfragF1 and AfragR1 also failed to produce a PCR product with template DNA extracted from mealybug, whiteflies, spider mites, aphids, thrips. *Botrytis cinerea*, *Xanthomonas campestris* pv. *zinniae* and the *Pseudomonas* spp. (Figure 4). Extracted DNA from the healthy host plants showed no cross-reactivity with primers AFragF1 and AFragR1 based on the failure to produce a PCR product with this primer set (Figure 5).

Sensitivity of the *A. fragariae*-specific PCR assay

DNA was successfully PCR-amplified from one individual *A. fragariae* nematode, extracted alone, or extracted together with increasing amounts of leaf tissue ranging from 1 to 20 leaf disks (20 leaf disks = 146 mg) removed from *A. nidus*, *Heuchera*, or *Dahlia* leaves. The PCR-assay using primers AfragF1 and AfragR1 also successfully detected a single nematode, up to the maximum of 1,000 pooled nematodes, in a background consisting of three *A. nidus* leaf disks (Figure 6). There was no DNA detected from the sample with 20 nematodes in three leaf disks, this was due to a complication with the extraction protocol for that sample. In further testing, the primers were able to detect 20 nematodes.

Morphological identification of nematodes

The four characteristics examined for each nematode isolate, including the large metacorpus, shape of head region, number of rows of oocytes, and number of tail mucro, were largely consistent among all nematode isolates collected with a few exceptions. The shape of the head region of the nematodes extracted from *C. wrightii* plants appeared more similar to *A. ritzembosi*. The shape of the tail region and the number of mucro on the tail region of nematodes extracted from *Chrysanthemum* 'Color Echo,' and *Dahlia* 'Bishop of Canterbury' deviated from original descriptions of *A. fragariae*, making the identification difficult. (Table 1) The shape of the head was variable among the *Aphelenchoides* isolates examined, and even at 1000× magnification, the tail mucro were difficult to discern. Interestingly, however, the *Aphelenchoides* DNA amplified with AFragF1/AFragR1 from naturally-infected *Cheilanthes wrightii*, *Chyrsanthemum* 'Color Echo,' and *Dahlia* 'Bishop of Canterbury,' was 169 bp and had 100% nucleotide identity with the corresponding 169-bp *A. fragariae* ITS1 sequence.

Detection of *A. fragariae* among plant samples

Over 112 foliar nematode-infected herbaceous and woody ornamental species and hybrids, from 25 different plant families, were collected across eight counties from three wholesale nurseries, one wholesale and retail nursery, four nursery and garden centers, one mail-order nursery, one retail outlet, and two landscape plantings in North Carolina (Table 1). Specimens of *Aphelenchoides* spp. were recovered from 116 of the plant samples that displayed symptoms of foliar nematode infection (Table 1). Based on morphology, 93 of these 116 isolates were identified as *A. fragariae*. Genomic DNA containing both plant and nematode DNA produced a 169-bp fragment when PCR-amplified with *A. fragariae* primers AFragF1 and AFragR1 for 100 of the 116 plant samples (Table 1). Nine of the plants that were identified based on morphological characteristics to be infected by *Aphelenchodies fragariae* using morphology were not positive using the species-specific primers (Table 1).

NaOH lysis as an alternative method for DNA extraction

The NaOH method of DNA extraction (1:100 dilution) was compared to DNA extracted using the Dneasy plant mini kit (Qiagen Inc., Valencia, CA) using duplicate plant samples (Figure 7). A dilution series of NaOH extracted DNA shows sensitivity ranging from 1:100 to 1:100,000. Nine nonsymptomatic plant samples were extracted using the Dneasy plant mini kit (Qiagen Inc., Valencia, CA) and the NaOH DNA extraction protocol. It was found that the DNA extracted using both the DNA extraction protocols failed to produce the 169-bp fragment when PCR amplified with the *A. fragariae* primers (AFragF1 and AFragR1). DNA extracted using both the NaOH method and the Dneasy plant mini kit (Qiagen Inc., Valencia, CA) from symptomatic plants samples of the same nine species as the nonsymptomatic plant samples, produced the expected 169-bp fragment when PCR

39

amplified with the *A. fragariae* primers (AFragF1 and AFragR1). A dilution series of NaOH

extracted DNA shows sensitivity ranging from 1:100 to 1:100,000. However, the NaOH

DNA extraction protocol has not been as extensively tested as the Dneasy plant mini kit

(Qiagen Inc., Valencia, CA) for sensitivity and reproducibility. However, NaOH DNA

extraction protocol is less time consuming and from preliminary testing is as accurate as the

Dneasy plant mini kit (Valencia Inc., CA), but more testing needs to be done.

DISCUSSION

The objective of this study was to develop a sensitive, objective, and reliable technique for the detection of the foliar nematode, *Aphelenchoides fragariae*, a common pathogen of ornamental plants in nurseries and greenhouses. The rDNA ITS1 sequence for *A. fragariae* was used to develop AfragF1/AfragR1 PCR primers that produced a 169 bp product specific to only *A. fragariae*. The sequences of seven *A. fragariae*, isolated from different geographical areas throughout North Carolina, showed that the DNA sequence amplified by the species-specific primers was 100% identical to the reported sequence and the sequence ITS1 DNA amplified from a known culture of *A. fragariae* using rDNA universal primers (Figure 2).

By using several isolates collected in NC, we demonstrated the utility of this newly developed primer pair for discriminating *A. fragariae*. The ITS1 sequence for *A. besseyi* and *A. ritzemabosi* were obtained from Sergei Subbotin at the University of California. Dr. Subbotin also provided DNA of *Aphelenchoides besseyi* for the use in testing the specificity of the selected primers. Based on BLASTn comparison of the *A. fragariae* sequence with both the *A. ritzemabosi* and *A. besseyi* ITS1 sequence (from S. Subbotin, UCR), the primers would not amplify a product from either of these species (Figure 2). The DNA extracted from *A. besseyi* did not produce an amplicon with primers AFrgaF1/AFragR1, demonstrating the specificity to only the *A. fragariae* species of foliar nematode. The inability of the AfragF1/AfragR1 primer sets to amplify a product from DNA of common host plants of *A. fragariae*, common pathogens of similar host plants, nor other nematode species tested provides further evidence of the specificity of the AfragF1/AfragR1 PCR assay to detect and identify *A. fragariae*.

The AfragF1/AfragR1 PCR-detection assay described here was found to be highly sensitive to minimal numbers of *A. fragariae*. The primers were able to detect a single *A. fragariae* nematode in a small amount (0.02 mg) of plant leaf tissue, as well as in a large amount (146 mg) of plant tissue. Typically, infected plants do not exhibit symptoms until the plant is heavily infested by nematodes, which can take months depending on the environmental conditions in the nursery or greenhouse. It may be necessary to sample a large amount of plant tissue in order to detect a plant that is not heavily infected. The dramatic increase in sensitivity of *A. fragariae* detection provided by the PCR-based assay provides the ability to identify foliar nematode infection in asymptomatic plant tissues and likely would reduce the need for large sample sizes. Conversely, a symptomatic heavily-infested leaf may have greater than 800 nematodes per three leaf disks (unpublished data), therefore by using 1000 nematodes in the same amount of tissue here, it can be concluded that a heavily infected leaf would also be detected using the PCR-based assay.

Specimens of *Aphelenchoides* spp. were recovered from 112 samples of different nursery plant species suspected of foliar nematode infection. Based on morphology, 93 of these isolates were identified as *A. fragariae*. Foliar nematodes from the remaining 19 plant samples either were not recovered from plant tissue using the water extraction method or the sample extracted using the water extraction method was not identified. Genomic DNA containing both plant and nematode DNA produced a 169-bp fragment when PCR-amplified with primers AFragF1 and AFragR1 for 100 of the 112 plant samples, including all water extracted samples identified as *A. fragariae* (Table 1). The data suggest that *A. fragariae* is by far the predominant foliar nematode species that infects many different ornamental plant

species and that the PCR-based assay is relatively efficient in detection of *A.fragariae*-infected plant samples.

Once plant tissues are received, multiple assays for foliar nematode detection can be completed within a day using the AfragF1/AfragR1 PCR detection assay without the need for clinic expertise in nematode identification. The DNA extraction using the Dneasy plant mini kit (Qiagen Inc., Valencia, CA) will take approximately two hours for 18 to 20 samples. High throughput kits are available for 96 samples, which can be done in about an hour. The PCR amplification and gel analysis will take approximately five hours. This protocol could be used in a plant disease diagnostic clinic where the grower is responsible for paying for the cost of the assays. Extraction of DNA from samples using the DNeasy plant mini kit (Qiagen Inc., Valencia, CA) is relatively expensive, however, especially if being used for foliar nematode sampling and detection on a large-scale basis. The NaOH DNA extraction technique was found to be very fast, simple, and reproducible. The NaOH DNA extraction from infected tissues can be performed in approximately 5 minutes for 18 to 20 samples, is inexpensive, and uses common laboratory reagents. In theory, coupling NaOH DNA extraction with the AfragF1/AfragR1 PCR protocol would be less cost prohibitive for diagnostic clinics to offer clientle. However, the NaOH technique of extraction has just recently been applied for the extraction of foliar nematode DNA and therefore has not been tested as extensively as the Dneasy plant mini kit (Qiagen, Inc., Valencia, CA) for the *A. fragariae* PCR assay.

Aphelenchoides fragariae can be transmitted over long distances in shipments of (often) asymptomatic infected plants, and once introduced, the foliar nematodes are able to spread throughout the recipient nursery with no economical effective method of control.

Exclusion and eradication of foliar-nematode infected plant material from a nursery is critical to management of the disease, and this ability requires an efficient foliar nematode detection assay. The current water extraction detection assay requires significant (often symptomatic) foliar nematode infection levels for detection, several days of nematode emigration from host tissues, and a trained clinician for accurate nematode identification. The *A. fragariae*-specific AFragF1/AFragR1 PCR assay developed here provides a sensitive, specific, and reliable means of detecting and identifying this foliar nematode species directly from infected host-plant tissue. The application of this *A. fragariae* PCR assay in plant disease diagnostic clinic use, and potential development of a real-time PCR assay based upon the results presented here, can provide an effective tool to improve foliar nematode management in ornamental plant nurseries.

LITERATURE CITED

Beckenbach, K., Blaxter, M., and Webster, J.M. 1999. Phylogeny of *Bursaphelenchus*
species derived from analysis of ribosomal internal transcribed spacer DNA
sequences. *Nematology*. 1(15): 539-548.

Cherry, T., Szalanski, A.L., Todd, T.C., and Powers, T.O. 1997. The internal transcribed
spacer region of *Belonolamius* (Nematoda: Belonolaimidae. *Journal of Nematology*.
29:23-29.

Chilton, N.B., Gasser, R.B., and Beveridge, I. 1995. Differences in a ribosomal DNA
sequence of morphology indistinguishable species within the *Hypodontus macropi*
complex (Nematoda: Strongyloidea). *International Journal for Parasitology*. 25: 647-
651.

Chizhov, V.N., Chumakova, O.A., Subbotin, S.A., and Baldwin, J.G. 2006. Morphological
and molecular characterization of foliar nematodes of the genus *Aphelenchoides: A.*
fragariae and *A. ritzemabosi* from the main botanical garden of the Russian Academy
of Sciences, Moscow. *Russian Journal of Nematology* 14:179-184.

Decker, H., 1989. Leaf- Parasitc Nematodes. In *Plant Nematodes and Their Control*.
Sveshnikova, N. M. Ed. Kinderbrook, NY. E.J. Brill Publishing Company, 354-358.

Fallas, G.A., Ferris, J.M., Faghihi, J. and Ireholm, A. 1994. Comparisons of isolates of
Heterodera avenae using 2-D PAGE protein patterns and ribosomal DNA. *Journal of*
Nematology. 26:144-151.

Fallas, G.A., Hahn, M.L., Fargette, M., Burrows, P.R., and Sarah, J.L. 1996. Molecular and
biochemical diversity among isolates of *Radopholus* spp. from different areas of the
world. *Journal of Nematology*. 28:422-430.

Harmey, J.H., and Harmey, M.A. 1993. Detection and Identification of *Bursaphelenchus* Species with DNA Fingerprinting and Polymerase Chain Reaction. *Journal of Nematology*. 25(3): 406-415.

Hunt, D.J. 1993. Aphelenchida, Longidoridae and Trichodoridae: Their Systematics and Biology. Northampton, UK: CAB International, 58-65.

Ibrahim, S.K., Perry, R.N., Burrows, P.R., and Hooper, D.J. 1994. Differentiation of Species and Population of *Aphelenchoides* and of *Ditylenchus angustus* Using a Fragment of Ribosomal DNA. *Journal of Nematology*. 26(4): 412-421.

Iwahori, H., Tsuda, K., Kanzaki, N., Izui, K., and Futai, K. 1998. PCR- RFLP and sequencing analysis of ribosomal DNA of Bursaphelenchus nematodes related to pine wilt disease. *Fundam. Appl. Nematol*. 21(6): 655-666.

Jagdale, G.B., and Grewal, P.S. 2002. Identification of alternatives for the management of foliar nematodes in floriculture. *Pest Management Science*. 58: 451-458.

Jagdale, G. B., and Grewal, P. S., 2004. Effectiveness of a Hot Water Drench for the Control of Foliar Nematodes *Aphelenchoides fragariae* in Floriculture. *Journal of Nematology*. 36(1):49-53.

Jagdale, G. B., and Grewal, P. S., 2006. Infection Behavior and Overwintering Survival of Foliar Nematodes, *Aphelenchoides fragariae*, on Hosta. *Journal of Nematology*. 38(1):130-136.

Joyce, S.A., Burnell, A.M., and Powers, T.O. 1994. Characterization of *Heterorhabditis* isolates by PCR amplification of segments of mtDNA and rDNA genes. *Journal of Nematology.* 25:260-270.

Maggenti, A. 1981. Aphelenchoid Parasites of Plant Shoots: Bud and Foliar Nematodes. In *General Nematology*. Starr, M.P. Ed. New York, NY USA: Springer-Verlag, 209-211.

Nasmith, C.G., Speranzini, D., Jeng, R., and Hubbes, M. 1996. RFLP analysis of PCR amplified ITS and 26Ss ribosomal genes of selected entomopathogenic nematodes (Steinernematidae, Heterorhibditidae). *Journal of Nematology*. 28:15-25.

Powers, T.O., and Harris, T.S. 1993. A Polymerase Chain Reaction Method for Identification of Five Major *Meloidogyne* Species. *Journal of Nematology*. 25(1): 1-6.

Powers, T. O., Todd, T. C., Burnell, A. M., Murray, P. C. B., Fleming, C. C., Szalanski, A. L., Adams, B. A., and Harris, T. S. 1997. The rDNA Internal Transcribed Spacer Region as Tazonomic Marker for Nematodes. *Journal of Nematology*. 29(4): 441-450.

Rozen, S. and Skaletsky, H. J. 2000. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Humana Press, Totowa, NJ, pp 365-386 Source code available at http://fokker.wi.mit.edu/primer3/.

Sanwal, K. C., 1961 A key to the species of the nematode genus Aphelenchoides Gischer, 1894. Canadian Journal of Zoology. 39:143-148.

Stevenson, L.A., Chilton, N.B., and Gasser, R.B., 1995. Differentiation of *Haemonchus placei* from *H. contortus* (Nematoda: Trichostrongylidae) by the ribosomal DNA second internal transcribed spacer. *International Journal of Parasitology*. 25:483-488.

Szalanski, A.L., Sui, D.D., Harris, T.S., and Powers, T.O. 1997, Identification of cyst nematodes of agronomic and regulatory concern by PCR-RFLP of ITS1. *Journal of Nematology*. 29:255-267.

Thiery, M., and Mugniery. 1996. Interspecific rDNA restriction fragment length polymorphism in *Globodera* species, parasites of solanaceous. *Fundamental and Applied Nematology*. 19: 471-479.

USDA Floriculture Crops 2005 Survey. 2006.
http://usda.mannlib.cornell.edu/reports/erssor/specialty/flo-bb/flo2005.pdf

Viran, T.C., Wakarchuk, D.A., Levesque, A.C., and Hamilton, R.I. 1992. Intraspecific rDNA restriction fragment length polymorphism in the *Xiphinema americanum* group. *Fundamental and Applied Nematology*. 15:563-573.

Viran, T.C. and McNamara, D.G. 1994 Potential for identification of quarantine nematodes by PCR. EPPO Bulletin 24: 453-458.

Wang, H., Qi, M., and Cutler, A. J. 1993. A simple method of preparing plant samples for PCR. Nucleic Acids Res. 21:4153-4154.

Wendt, K.R., Swart, A., Varian, T.C., and Webster, J.M. 1995. *Ditylenchus africanus* sp. n. from South Africa—a morphological and molecular characterization. *Fundamental and Applied Nematology*. 18:241-250.

Zijljstra, C., Uenk, B.J., and Van Silfhout, C.H. 1997. A reliable, precise method to differentiate species of root-knot nematodes in mixtures on the basis of ITS-RFLPs. *Fundamental and Applied Nematology*. 20:59-63.

Zijlstra, C., Lever, A.E.M., Uenk, B.C., and Van Silfhout, C.H. 1995. Differences between

ITS regions of isolates of root-knot nematodes *Meloidogyne hapla* and *M. chitwoodi*.

Phytopathology. 85:1231-1237

Table 1. Assay of 121 herbaceous and woody ornamental species and hybrids for foliar nematode infection collected across eight counties from three wholesale nurseries, one wholesale and retail nursery, four nursery and garden centers, one mail order nursery, one retail outlet, and two landscape plantings in North Carolina. Plant tissue incubation in water and morphological identification of extracted nematodes by microscopy and a PCR-based assay to detect species-specific ITS rDNA were used for confirmation of plant infection by the foliar nematode, *Aphelenchoides fragariae*. (Legend: Different Sequenced: Sample was morphologically different than A. fragariae morphology and was sequenced NO ID: The extracted sample was not identified morphologically. NA: The DNA extracted from the sample was used in the PCR-based assay).

Family Name	Host	Origin	Date of Collection	*A. fragariae* Morphology	PCR
Anemiaceae	*Anemia tomentosa*	PDN	11/19/2004	+	+
Apiaceae	*Angelica gigas*	PDN	11/08/2004	+	-
	Angelica gigas	PDN	11/19/2004	+	-
Aristolochiaceae	*Asarum canadense*	PDN	07/28/2005	+	+
Asteraceae	*Asarum maximum* 'Ling Ling'	PDN	11/19/2004	+	+
	Chrysanthemum 'Color Echo'	PDN	07/28/2005	Different Sequenced	+
Berberidaceae	*Chrysanthemum* 'Jettie'	PDN	12/03/2004	+	-
	Dahlia 'Bishop of Canterbury'	PDN	07/28/2005	Different Sequenced	+
	Echinacea 'Paranoia'	PDN	08/08/2005	+	+
	Echinacea purpurea 'Sparkler'	PDN	11/08/2004	+	-
	Heliopsis helianthoides 'Loraine Sunshine'	PDN	11/08/2004	+	+
	Marshallia caespitosa	PDN	08/18/2005	+	+
	Podophyllum pleianthum	PDN	07/28/2005	+	+
Boraginaceae	*Brunnera macrophylla* 'Jack Frost'	PDN	07/28/2005	+	+
Buddlejaceae	*Brunnera macrophylla* 'Looking Glass'	PDN	6/15/2006	+	+
	Buddleja davidii 'Bonnie'	PDN	07/28/2005	+	+
Caprifoliaceae	*Buddleja davidii* 'Nanho Purple'	JNC	5/2006	NO ID	+
	Buddleja davidii 'Petite Plum'	FGC	07/28/2004	+	+
	Abelia sp.	HN	10/21/2004	+	+

50

Table 1 (Continued)

Family Name	Host	Origin	Date of Collection	*A. fragariae* Morphology	PCR
Dryopteridaceae	*Abelia* × 'Edward Goucher'	Lowes	10/30/2004	NO ID	-
	Abelia grandiflora 'Little Richard'	JN	07/29/2004	+	+
Gesneriaceae	*Athyrium filix-femina* 'Frizelliae'	PDN	11/08/2004	+	-
	Athyrium filix-femina 'Lady in Red'	PDN	12/03/2004	+	+
Iridaceae	*Athyrium otophorum*	BB	02/10/2006	+	+
	Diplazium pycnocarpon	PDN	07/28/2005	+	+
Lamiaceae	*Dryopteris affinis*	BB	02/10/2006	NO ID	+
	Dryopteris atrata	BB	02/10/2006	NO ID	+
	Dryopteris carthusiana	PDN	05/20/2005	+	+
	Dryopteris celsa	BB	02/10/2006	NO ID	+
	Dryopteris dilatata 'Lepidota Cristata'	PDN	11/19/2004	+	+
	Dryopteris dilitata 'Recurvata'	BB	02/10/2006	NO ID	+
	Dryopteris filix-mas	BB	02/10/2006	NO ID	+
	Dryopteris filix-mas 'Barnesii'	BB	02/10/2006	NO ID	+
	Dryopteris filix-mas 'Linearis Polydactyla'	PDN	08/16/2005	+	+
	Dryopteris goldiana	PDN	12/13/2004	+	+
	Dryopteris fuscipes	PDN	12/12/2005	+	+
	Dryopteris lorentzii	PDN	11/19/2004	+	-
	Dryopteris marginalis	BB	02/10/2006	NO ID	+
	Dryopteris remota	BB	02/10/2006	+	+
	Polystichum	PDN	07/29/2004	-	+
	Polystichum acrostichoides	PDN	07/29/2004	-	+
	Polystichum xiphophyllum	PDN	05/27/2005	+	+
	Gloxinia nematanthodes 'Evita'	PDN	12/12/2005	+	+
	Iris ensata 'Variegata'	TNT	11/02/2004	+	+
	Sisyrinchium palmifolium	PDN	11/08/2005	+	+
Liliaceae	Agastache *'Blue Fortune'*	*PDN*	08/16/2005	+	+

Table 1 (Continued)

Family Name	Host	Origin	Date of Collection	*A. fragariae* Morphology	PCR
Liliaceae	*Agastache foeniculum* 'Golden Jubilee'	PDN	12/12/2005	+	+
	Lamium galeobdolon 'Petit Point'	PDN	12/03/2004	+	+
	Lamium maculatum 'Orchid Frost'	PDN	11/19/2004	+	+
	Salvia 'Indigo Spires'	PDN	08/08/2005	+	+
	Salvia 'Maraschino'	PDN	12/12/2005	+	+
	Salvia cacaliaefolia	BB	02/10/2006	NO ID	N/A
	Salvia chiapensis	BB	02/10/2006	NO ID	N/A
	Salvia farreri var. *glechomifolia*	BB	02/10/2006	NO ID	N/A
	Salvia microphylla 'San Carlos Festival'	PDN	08/08/2005	+	+
	Salvia uliginosa	PDN	08/18/2005	+	+
	Disporum smilacinum 'Aureovariegata'	PDN	5/2006	+	+
Osmundaceae	*Hosta*	FC	07/15/2004	+	+
	Hosta	RRGN		+	+
	Hosta 'Christmas Candy'	PDN	07/28/2005	+	+
	Hosta 'Ebb Tide'	PDN	08/18/2005	+	+
	Hosta 'Golden Tiara'	JNC	2006	NO ID	+
	Hosta 'Lemon Lime'	PDN	08/08/2005	+	+
	Tricyrtis 'Moonlight Treasure'	PDN	7/28/2005	+	+
	Tricyrtis hirta 'Moonlight'	PDN	6/15/2006	+	+
	Tricyrtis macrantha	PDN	06/21/2005	+	+
	Trillium rugelii	PDN	07/28/2005	+	+
	Osmunda japonica	PDN	07/28/2005	+	+
Oxalidaceae	*Osmunda regalis*	PDN	11/08/2004	+	+
	Osmunda regalis	BB	02/10/2006	NO ID	+
	Oxalis regnellii 'Aureovariegata'	PDN	5/2006	+	+
Plumbaginaceae	*Ceratostigma willmottianum* 'Palmgold'	PDN	08/18/2005	+	+
Polygonaceae	*Tovara virginiana* 'Painters Palette'	PDN	07/28/2005	+	+
Polypodiaceae	*Pteris sp.*	PDN	08/16/2005	+	+

Table 1 (Continued)

Family Name	Host	Origin	Date of Collection	*A. fragariae* Morphology	PCR
Pteridaceae	*Pyrrosia lingua ex.* 'Tachiba Koryu'	PDN	12/13/2004	+	+
	Cheilanthes alabamensis	PDN	04/05/2005	+	+
Ranunculaceae	*Cheilanthes argentea*	PDN	08/18/2005	+	+
	Cheilanthes lanosa	PDN	06/21/2005	+	+
	Cheilanthes wrightii	PDN	07/28/2005	Different Sequenced	+
	Mildella nitidula	PDN	6/15/06	+	+
	Pellaea nitidula	PDN	12/03/2004	+	+
	Pellaea nitidula	PDN	07/28/2005	+	+
	Anemone hupehensis 'Crispa'	PDN	07/28/2005	+	+
Rosaceae	*Anemone hupehensis* 'Pamina'	BB	2/2006	+	+
	Anemone 'Prinz Heinrich'	BB	2/2006	+	+
	Rubus taiwanicola	PDN	05/15/2004	+	+
Saxifragaceae	Fragaria JC Barnham Strawberry Leaves	PDIC	05/15/2005	+	+
	Bergenia 'Bressingham Ruby'	PDN	06/21/2005	+	+
Scrophulariaceae	*Heuchera* 'Frosted Violet'	PDN	08/16/2005	+	+
	×Heucherella 'Birthday Cake'	PDN	11/08/2004	+	+
	×Heucherella 'Party Time'	PDN	07/28/2005	+	-
	×Heucherella 'Party Time'	PDN	12/03/2004	+	-
	×Heucherella 'Sunspot'	PDN	07/28/2005	+	+
	×Tiarella 'Butterfly Wings'	PDN	07/28/2005	+	+
	Tiarella cordifolia 'Oconee Spreader'	PDN	6/2006	+	+
	Tolmiea menziesii 'Cool Gold'	PDN	05/15/2005	+	+
	Digitalis sp. (Foxglove)	IN	10/21/2004	+	+
Thelypteridaceae	*Veronica spicata* 'Royal Candles'	PDN	07/28/2005	+	+
	Thelypteris dentata	PDN	07/28/2005	+	+

Table 1 (Continued)

Family Name	Host	Origin	Date of Collection	*A. fragariae* Morphology	PCR
Verbenaceae	*Lantana camara*	TNT	11/02/2004	+	+
	Lantana camara 'Miss Huff'	TNT	11/02/2004	+	+
	Lantana camara 'Miss Huff'	JNC	11/2006	NO ID	+
	Lantana camara 'New Gold'	JN	05/15/2005	+	+
	Lantana camara 'New Gold'	WG	07/29/2004	NO ID	+
	Verbena 'Blue Princess'	PDN	08/08/2005	+	+
	Verbena 'Hot Lips'	PDN	12/03/2004	+	+
	Verbena 'Snowflurry'	PDN	12/13/2004	+	+
	Verbena 'Snowflurry'	BB	02/10/2006	NO ID	+
	Verbena candensis	PDN	07/28/2005	+	+
	Verbena tenuisecta 'Decked Out'	PDN	04/05/2005	+	+

Plate A:

Plate B:

Metacorpus

Head Region

Plates C:

Plate D:

Oocytes

Tail Region
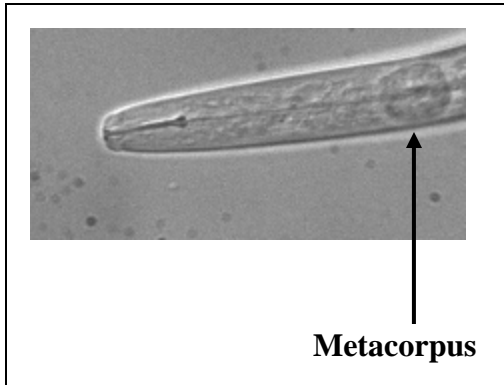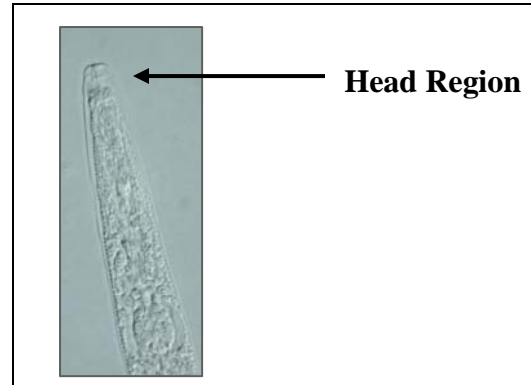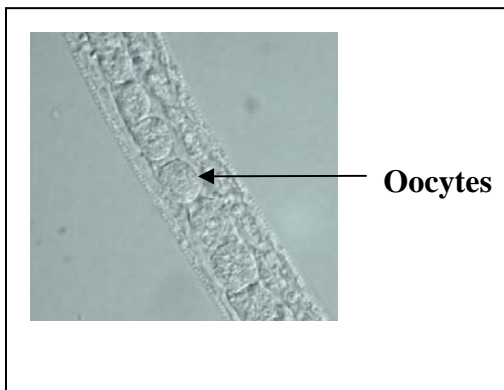
Figure 1. Four characteristics examined under a light microscope to identify the foliar nematode *Aphelenchoides fragariae*. Plate A: A characteristic of the Order *Aphelenchida* is a large well-developed metacarpus extending almost the entire width of the nematodes body. Plate B: The cephalic (head) region of *Aphelenchoides fragariae* is relatively high and almost continuous with the body. Plate C: The oocytes in female *A. fragariae* nematodes are in a single row. Plate D: The tail region for *A. fragariae* ends in a blunt point.
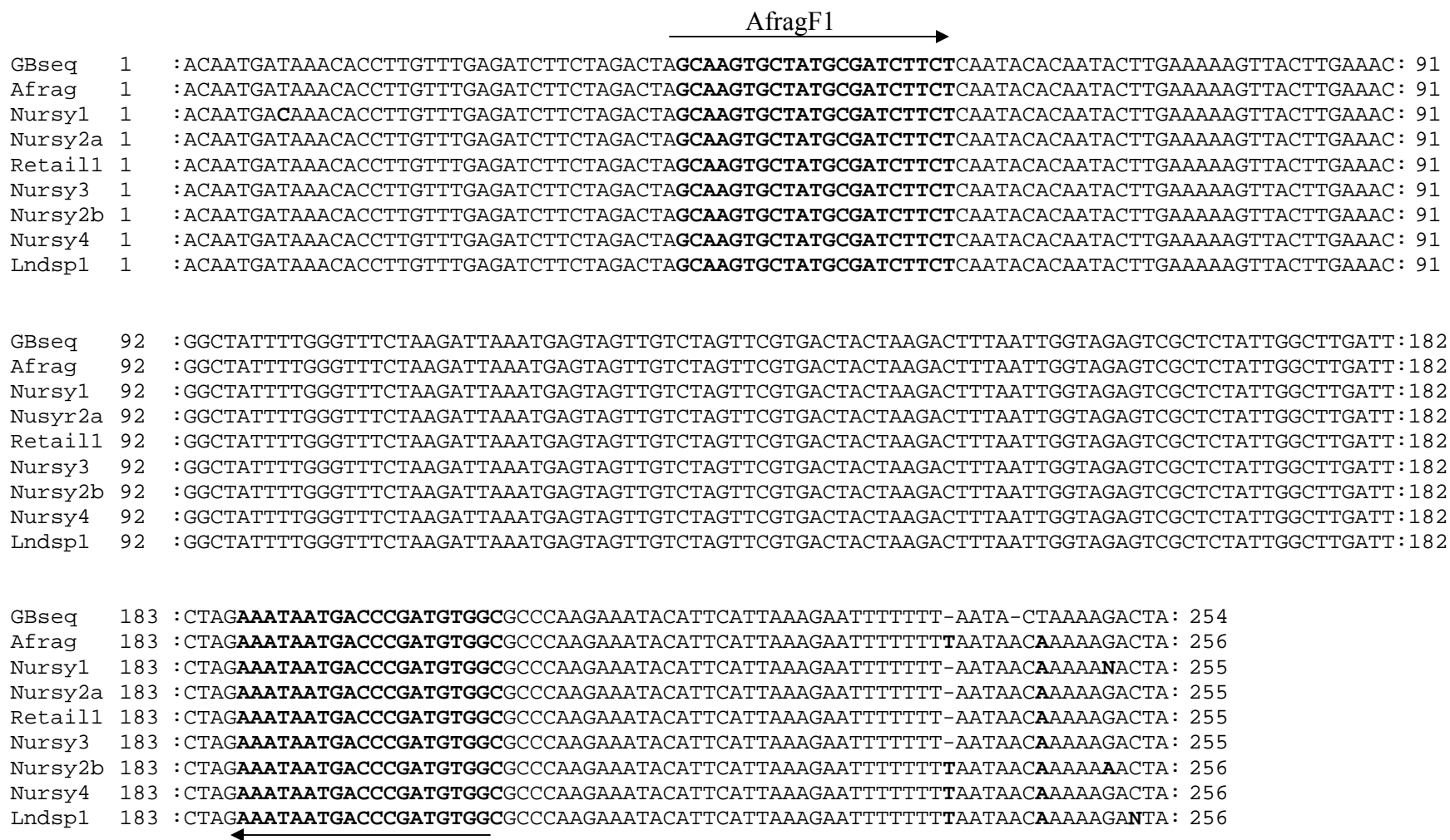
Figure 2. Nucleotide sequence alignment of the ITS1 region of rDNA among nine *A. fragariae* isolates (GBseq = GenBank Accession No. AF119049; Afrag = monoxenic culture). Bold type with arrows represent primer annealing sites. Nucleotide sequence differences are in bold type. Gaps introduced to maximize alignment are marked by hyphens.
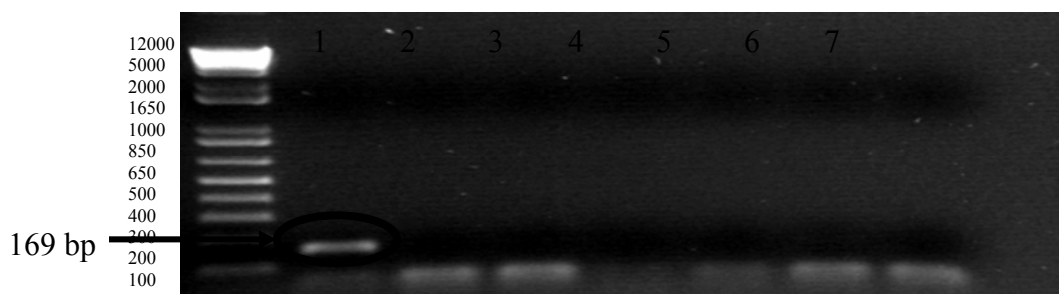
Figure 3. Species-specific PCR primers (AfragF1 and AfragR1) only produced the expected amplicon of 169 bp using DNA extracted from (Lane 1) *Aphelencohides fragariae* but not using DNA extracted from (Lane 2) *Meloidogyne incognita,* (Lane 3) *Heterodera schachtii,* (Lane 4) *Pratylenchus penetrans*, (Lane 5) *Ditylenchus dipsaci*, (Lane 6) *Caenorhabditis elegans*, (Lane 7) Water Negative Control and (Not Pictured) *Aphelenchoides besseyi,* or using DNA from an unidentified *Aphelenchoides* sp. collected in Costa Rica.
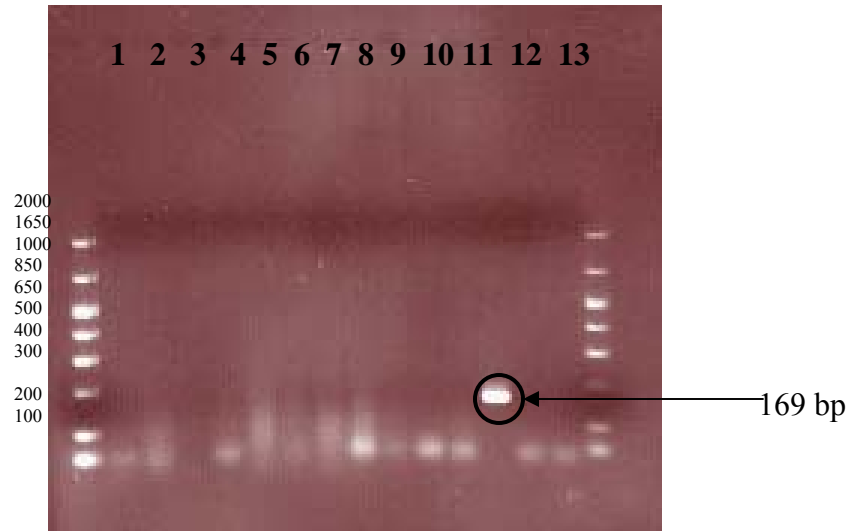
Figure 4. Species-specific PCR primers (AfragF1 and AfragR1) failed to produce a PCR product with template DNA extracted from the common greenhouse and nursery pests and pathogens including (1) mealybug (*Pseudococcus affinis*), (2) *Xanthomonas campestris* pv. *Zinniae*, (3) Spider mites (*Tetranychus* sp.), (4) whiteflies (*Trialeurodes* spp.), (5)*Botrytis cinerea*, (6) *Pseudomonas* sp., (7) *Botrytis cinere*, (8) *Pseudomonas* sp., (9) spidermites (*Tetranychus* sp.), (10) aphids (*Microsiphum* spp. and *Myzus persicae*), (11) *Xanthomonas campestris* pv. *Zinniae*, (12) whiteflies (*Trialeurodes* spp.), (13) *Aphelenchoides fragariae* Positive Control, (14) Water Negative Control. (15) thrips (*Frankliniella occidentalis*).
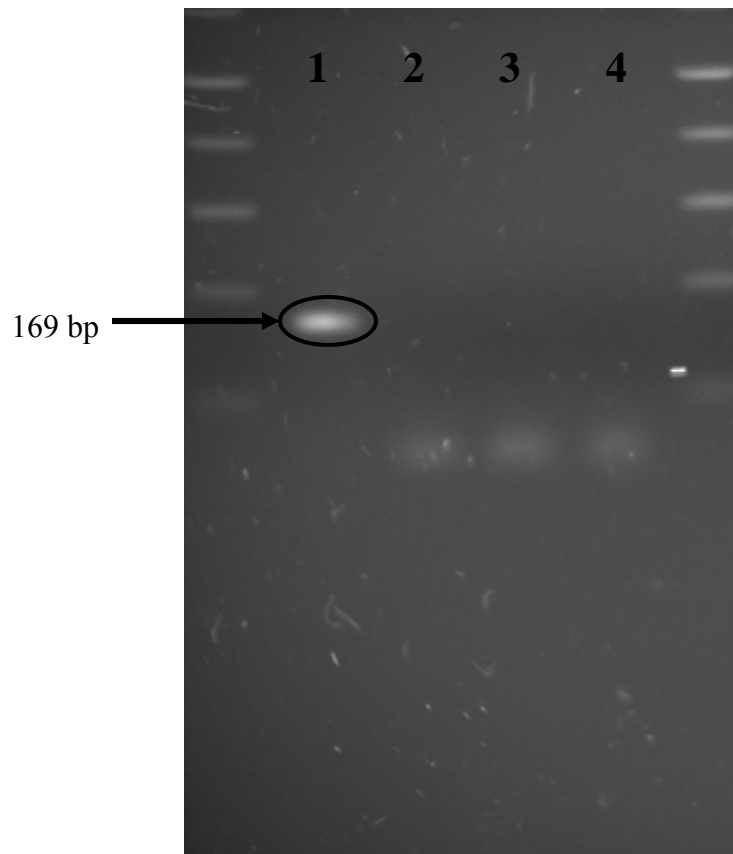
Figure 5. DNA extracted from healthy host plants produced no amplicon with the species specific PCR primers (AfragF1 and AfragR1). (1) *Aphelenchoides fragariae*, (2) Water Negative Control, (3) *Lantana* 'New Gold', (4) *Salvia* microphylla. Healthy *Verbena* 'Snowflurry', *Heuchera* 'Frosted Violet', *Dahlia* 'Bishop of Canterbury' and *Asplenium nidus* also produced no amplicon with the AfragF1 and AfragR1 primers but are not pictured here.

Number of Nematodes per 3 six mm Leaf Disks
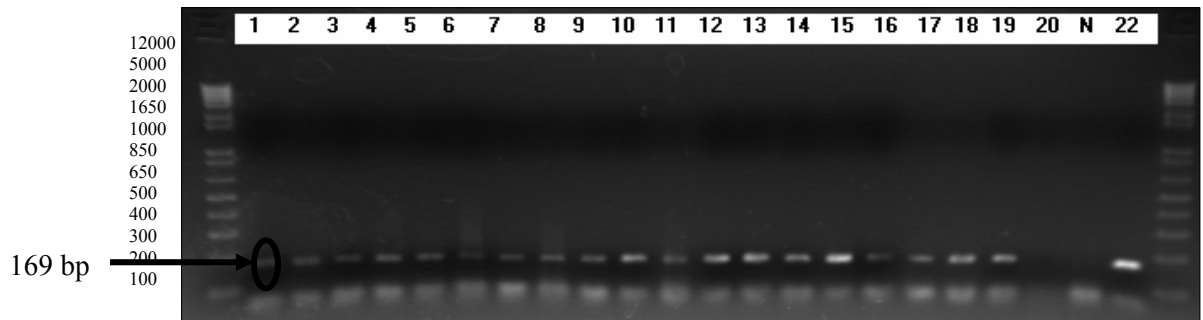
Figure 6. The predicted amplicon of 169 bp was produced from ITS1 rDNA from different numbers of *A. fragariae* ranging from 1 to 1,000 nematodes mixed with DNA extracts of three, six-mm *Asplenium nidus* leaf disks using the species-specific PCR primers (AfragF1 and AfragR1).
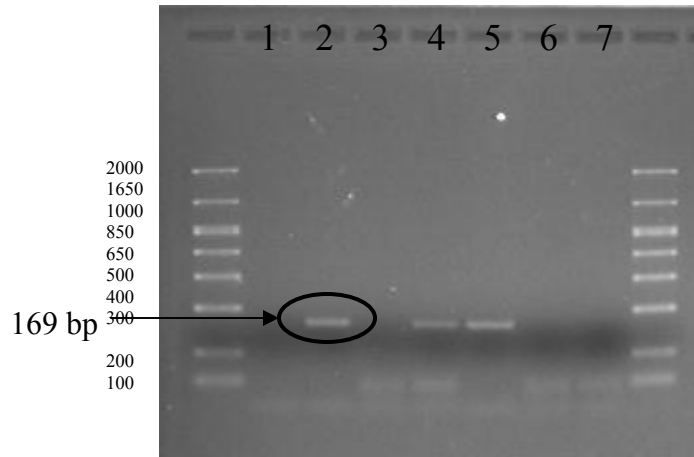
Figure 7. A NaOH method of DNA extraction was compared to DNA extracted using the Qiagen Dneasy plant mini kit (Qiagen Inc., Valencia, CA) using the same plant samples. Lane 1: Healthy *Asplenium nidus* Dneasy extraction; Lane 2: Symptomatic *A. nidus* Dneasy Extraction; Lane 3: Healthy *A. nidus* NaOH extraction; Lane 4: Symptomatic *A. nidus* NaOH Extraction; Lane 5: Positive *A. fragariae* Dneasy control; Lane 6: Negative NaOH Control; Lane 7: Negative H_2O Control.

CHAPTER 2. DEVELOPMENT OF A SAMPLING PROTOCOL FOR ORNAMENTAL NURERIES FOR USE WITH A PCR-BASED DIAGNOSTIC ASSAY FOR THE DETECTION OF *APHELENCHOIDES FRAGARIAE*

ABSTRACT

The foliar nematode species, *Aphelenchoides fragariae*, is one of three plant-pathogenic foliar nematode species of economic importance in the ornamental horticulture industry that causes millions of dollars in commercial plant damage each year within and outside the United States. This is due in part to the aesthetic damage foliar nematode infection causes in plant tissue. *Aphelenchoides fragariae* have been reported to infect over 250 species of ornamental foliage plants, as well as a large number of perennials, bedding, foliage, and woody ornamentals. Foliar nematodes cause chlorotic and necrotic lesions that typically follow the venation patterns of the leaf. Plant symptoms generally do not appear until the plant is heavily infected with nematodes, making early detection problematic. The traditional method of foliar nematode detection is a water incubation extraction assay. This assay requires the nematode to migrate out of the leaf tissue into surrounding water and be identified under a microscope. The water incubation extraction assay is often difficult, time-consuming, and requires someone trained to identify foliar nematodes. Foliar nematodes may not be recovered from plant samples that harbor low populations of nematodes using the water incubation extraction assay, presenting a limitation in assay sensitivity. A PCR-based diagnostic assay has been developed for the detection of *A. fragariae* using the species-specific primers AFragF1/AFragR1 (see Chapter 1). The PCR-based diagnostic assay was

found to be highly sensitive to one *A. fragariae* nematode in up to 146 mg of background leaf tissue. The most effective means of foliar nematode control is exclusion from plant nurseries. Millions of ornamental plants are produced and sold from commercial nurseries each year many of which are susceptible to infection by foliar nematodes. There is an increasing concern among growers that asymptomatic foliar nematode-infected plant material is currently being sold and transported across nursery, state, and national boundaries. A preliminary sampling protocol that could be used with a sensitive diagnostic assay to detect foliar nematodes in asymptomatic plants was developed. The PCR-based assay detected significantly more (p<0.05) more nematodes in asymptomatic leaf tissue as compared to the water incubation extraction assay. For leaves with obvious symptoms of foliar nematode infection, both methods were statistically equivalent in detecting nematodes in leaf tissue. Both the PCR assay and the water extraction technique were compared in split samples to assess foliar nematode distribution within plant architecture and within blocks of different plant species grown in commercial nurseries. Although foliar nematodes were detected in all parts of *Verbena 'Snowflurry'* plants, the majority of the nematodes were located in the lower third of the plant. A sampling of mixed *Anemone* spp., plants confirmed that the PCR-based assay detected foliar nematode infection in a greater number of plants than the water incubation extraction assay. The *A. fragariae* PCR assay was significantly more sensitive than the water extraction assay in detecting foliar nematode infection of plants in separate blocks of *Disporum smilacinum* and *Oxalis regnellii,* but heavy infection of a block *Mildella nidulata* plants by foliar nematodes did not indicate a difference between detection assays. Application of Taylor's Power Law on nematode counts from water extraction assays of nursery blocks of three plant species suggested a mostly aggregated distribution of foliar

nematodes within each block and optimal sample sizes of 23, 38, and 41 plants depending

upon plant species. The coupling of the increased sensitivity and specificity of the PCR-

based assay with assessment of nematode distribution within plants and nursery facilities will

provide a critical tool for management decisions to reduce foliar nematode damage to

ornamental nursery plants.

INTRODUCTION

The foliar nematode species, *Aphelenchoides fragariae,* is responsible for millions of dollars in plant disease losses each year in the ornamental nursery industry (Jagdale and Grewal, 2006). This is due in part to the aesthetic damage the nematodes cause to the plant tissue. *Aphelenchoides fragariae* have been reported to infect over 250 species of ornamental plants including *Lamium*, *Hostas*, *Aster*, *Athyrium*, *Dahliah*, *Anemone*, *Oxalis*, and numerous ferns, grown in commercial nurseries and landscapes throughout the world (Decker 1989; Jagdale and Grewal, 2006).

Current research suggests that *A. fragariae* may overwinter as juveniles and adults in the soil, dry infected leaves, and dormant crowns, but not in the roots of infected plants (Jagdale and Grewal, 2006). During the spring, the nematodes become active and begin to migrate from the soil and infected leaf debris up the stems and petioles in thin films of water to the leaves (Buckley and Gould, 2003; Jagdale and Grewal 2006; Wallace, 1959) The nematodes enter the leaf tissue through the stomata on the underside of the leaf as well as through wounds on the leaf surface (Decker 1989; Wallace, 1959). Once the nematodes infect the leaf, they feed upon the mesophyll and parenchyma tissues and induce angular shaped lesions due to the inability of the nematode to cross leaf veins when inside the tissues. The nematodes much emerge from leaves in films of water to cross leaf veins and infect new leaf tissues. Hundreds of foliar nematode eggs are laid in the leaf mesophyll, and at an optimal temperature of 18°C, the nematodes will mature into reproductive adults within 10 to 11 days (Decker, 1989). If conditions remain favorable for nematode development and reproduction, many generations occur rapidly resulting in a heavy infestation (Decker, 1989).

Occasionally, the entire leaf will become infected, causing the leaf to dry and drop

prematurely, affecting the marketability of the plant. Symptoms can appear as chlorotic

angular lesions, which will slowly become necrotic (De Maeseneer, 1964; Mor, 1993;

Sanwal, 1959). Symptoms may also include discoloration, distorting, and dwarfing of the

leaves, stems, flowers, or bulbs (LaMondia, 1999). Typically, infected plants do not exhibit

symptoms until the plant is heavily infected by nematodes, which can take months depending

on the environmental conditions in the nursery or greenhouse. The most favorable conditions

for symptom development occur in the spring and early summer, but symptom development

slows throughout the summer (Decker, 1989).   Symptom development will vary, however,

based upon host plant species the environment of the nursery or greenhouse.

Foliar nematode infestation can quickly spread throughout a growing facility by

means of infected leaves contacting uninfected plant tissue, splash dispersal, and natural or

mechanical movement (Daughtrey et al., 1995; Decker, 1989; LaMondia, 1999). Once foliar

nematodes become established in a nursery or greenhouse, management to reduce plant

damage and nematode spread is difficult due to the lack of available and effective control

strategies. Several nematicides, insecticides, and miticides are effective for foliar nematode

suppression, however the US Environmental Protection Agency (EPA) has restricted or

banned the use of the majority of the effective nematicides for foliar nematode control

(Jagdale and Grewal, 2004, 2002; Johnson and Gill, 1975; LaMondia, 1999). The nematicide

bans and restrictions are due to environmental pollution concerns and human health risks

(Schulze, 2003). Some non-chemical treatment strategies have also been developed to reduce

foliar nematode infection of plants. A hot water dip is routinely used for the control of foliar

nematodes in bare-rooted plants, dormant crowns, bulbs, and runners of plants important in

the ornamental horticulture industry. This method is time consuming and labor intensive, so a more efficient hot water drench has been evaluated on foliar nematode-infected (*Hosta* sp) and fern (*Matteuccia pensulvanica*) (Jagdale and Grewal, 2004). The hot water drench was found to reduce the foliar nematode population but not eradicate them. Even though these methods are able to reduce the foliar nematode population, it is essential for long-term management to establish good sanitation practices throughout the greenhouse or nursery to minimize introduction and spread. This includes minimizing leaf wetness, isolating or removal of infected plants, removing infested leaf litter throughout the greenhouse, and sanitizing cutting utensils, benches and floors with chlorine bleach (Dunn, 2005; LaMondia, 1999). The best foliar nematode management strategy is to use nematode-resistant plant cultivars if available or use nematode-free planting stock, propagation materials, and mother plants (Dunn, 2005; Thorne, 1961).

Millions of ornamental plants are produced and sold from commercial nurseries each year, many of which are susceptible to infection by foliar nematodes (USDA, 2006). There is an increasing concern among growers that foliar nematode infected plants are being sold and transported across nursery, state, and national boundaries (Jagdale and Grewal, 2006). Unfortunately, many infected plant shipments arrive without showing symptoms and are placed the nursery with previously uninfected plants providing the opportunity for foliar nematodes to spread throughout the nursery. An efficient and sensitive diagnostic assay and sampling protocol that could be used to detect foliar nematodes in asymptomatic plants would be a significant advance in facilitating effective control.

Currently, a water incubation extraction assay is used to detect the presence of foliar nematodes in infected plant tissue. For the water incubation extraction assay, the leaf

67

specimens are placed in water at room temperature for 24 to 48 hours to allow the nematodes to migrate out of the foliage and be collected in the surrounding water (Jagdale and Grewal, 2002; Volvas et al, 2005). Extracted nematodes are then identified under a microscope by a trained specialist using morphological characteristics that are unique to each nematode species. The fact that foliar nematodes may not be recovered from plant samples that harbor low populations of nematodes using the water incubation extraction assay presents problems with the current assay's sensitivity. A PCR-based diagnostic assay has been developed for the detection of *Aphelenchoides fragariae* using species-specific primers developed inside the ITS1 region of the ribosomal DNA (McCuiston, 2007). For the PCR-based diagnostic assay, total DNA is extracted directly from nematode-infected plant material and used in a PCR reaction to yield a 169-bp PCR product if *A. fragariae* are present in the plant material. The PCR primers were found to be able to detect a single foliar nematode within three plant leaf disks and are specific to only the species *Aphelenchoides fragariae* (McCuiston, 2007).

It is neither practical nor economically feasible to sample every susceptible host plant within a nursery, greenhouse, or plant shipment to detect the presence of foliar nematodes. Therefore, a sampling protocol is necessary to maximize the chance of detecting nematodes if present, but to minimize the number of plants that must be sampled in order to detect an *A. fragariae*-infected plant. The majority of the sampling protocols that have been developed for are for root pathogenic plant-parasitic nematodes. Root pathogenic nematodes are sampled for many reasons including to determine the spatial distribution of the nematodes throughout the field or soil column, for risk advisories based on population densities, management practices, and damage assessment (Abd-Elgawad and Hasabo, 1995; Duncan et al., 1994; Kashaija et al., 2004). For most of the root pathogenic nematodes sampling

protocols, soil cores are collected systematically throughout the field or sampling unit.

Nematodes are extracted from soil using a nematode extraction technique such as a

Baermann funnel or elutriation (Boag, 1984; Duncan, 1994; Kashaija et al, 2004; Singh and

Sitaramaiah, 1994). However, the sampling protocols vary based on the crop, species, and

biology of the nematode being sampled, the relationship between host and the nematode, and

the soil type (Singh and Sitaramaiah, 1994). Since *Aphelenchoides spp*. primarily inhabit the

foliage of the plant, it is necessary to obtain and analyze leaf samples. Currently, there is no

established sampling protocol for the detection of foliar nematodes in infected plant tissue or

within a group of plants. In order to develop an accurate and sensitive protocol five

fundamental concepts need to be considered. First is to develop an understanding of the

nematode's basic biology. Second, the distribution of the nematodes within the plant must be

determined, which has not been extensively studied with foliar nematodes. The third concept

is that sampling efficiency protocols must balance both economics and accuracy to a set an

acceptable degree of precision (Southwood and Henderson, 2000). The fourth concept is an

understanding of the spatial distribution of foliar nematodes within the sampling area.

Finally, a precise sampling size must be determined for the sampling plot to provide a

representative sample (Southwood and Henderson, 2000). The spatial distribution and

sample size can be determined empirically using one of several mathematical distributions,

including Taylor's Power Law (Taylor, 1961). Taylor's Power Law uses the relationship

between the mean and variance to determine the dispersion patterns or the degree of

aggregation of the target population within the sampling area. Taylor's Power Law has been

effectively used to characterize the spatial distribution and develop a sampling size of several

root pathogenic plant-parasitic nematodes (Abd-Elgawad and Hasabo, 1995; Boag and

Topham, 1984; Kashaija et al., 2004; Perry, 1983). An algorithm derived from Taylor's Power Law, using the calculated Taylor's power law coefficients, has been developed to provide an appropriate sampling size per sampling plot with a defined degree of precision (Wilson et al., 1983). We hypothesize that Taylor's Power Law could, therefore, provide an accurate assessment of the degree of aggregation and sample size foliar nematodes in a nursery block of plants.

The objective of this study was to assess foliar nematode distribution and determine optimal sampling size, using Taylor's Power Law, that can be used to sample propagative stock or incoming and existing plants for the presence of foliar nematodes. Foliar nematodes cause different symptoms based on the differences in leaf and plant architecture (Thorne, 1961). Therefore, the location of the nematodes within the plant may vary based on the architecture of each plant species. Blocks of five different plant species showing signs of foliar nematode infection, *Verbena* 'Snowflurry', *Anemone* spp., *Oxalis regnellii*, *Disporum smilacinum* 'Aureovariegata', and *Mildella nitidula*, were sampled over time to assess the distribution and degree of foliar nematode infestation. Each of the samples was evaluated using both the traditional water extraction assay and the PCR-detection assay for foliar nematode identification. A t-test was used to determine, which of the assays is the most sensitive and would be the most effective in detecting the presence of foliar nematodes within the nursery block. Taylor's Power Law was used to determine the degree of aggregation of the foliar nematodes within the nursery blocks and to determine sample sizes to detect foliar nematodes infecting *O. regnellii*, *D. smilacinum*, and *M. nitidula* with economically-acceptable levels of precision.

MATERIALS AND METHODS

Detection of Foliar Nematodes in Plant Samples

Blocks of *Verbena* 'Snowflurry', *Anemone* spp., *Disporum smilacinum, Oxalis regnellii*, and *Mildella nitidula* plants from ornamental plant nurseries showing symptoms of foliar nematode infestation were used as test plants for all nematode detection assays. Six disks were removed from each leaf sampled and foliar nematodes were extracted from three leaf disks of a split sample using the water extraction method (Jagdale and Grewal, 2002; Vovlas et al., 2004). The leaf disks were placed in a small 60X15 mm Petri dish with just enough water to completely cover the leaf disks. The plant tissue samples in water were incubated at room temperature for 24 hours and the nematodes that emigrated from the tissues were identified and counted under a microscope.

The three remaining leaf disks per each split sample were analyzed for the presence of foliar nematodes using a foliar nematode-specific PCR assay designed to obtain a 169 bp product amplified specifically from the ITS1 region of *A. fragariae* rDNA (McCuiston, 2007). Leaf tissue in a 2-ml conical tube containing two 5 mm glass beads was placed in liquid nitrogen for 30 seconds and then pulverized three times in a mini bead beater for 10 seconds at 2500 rpm. Isolation of DNA from each sample extract was subsequently performed using a Dneasy Plant Mini Kit (Qiagen Inc., Valencia, CA). The foliar nematode PCR reaction mixture included 2 µl of the extracted DNA, 2.5µl of 10X PCR buffer, 0.5µl of 50µM $MgCl_2$, 0.5µl of 10µM dNTPs, 1.0µl a 10µM ITS1 AfragF1 forward primer (GCAAGTGCTATGCGATCTTCT), 1.0µl a 10µM ITS1 AfragR1 reverse primer (GCCACATCGGGTCATTATTT), 0.25 µl of Platinum Taq DNA polymerase (Invitrogen Inc., Carlsbad, CA) and sterile molecular grade distilled water to a volume of 25µl. The

reaction was performed in a Geneamp PCR System 2700 thermocycler (Applied Biosystems, Foster City, CA) with the following program: 94 ºC for two minutes, 39 cycles with each including 94ºC for one minute, 53º for 40 seconds, 72 ºC for one minute, and a final cycle at 72 ºC for ten minutes. The thermocycler was set to cool and hold at 4 ºC after the program was complete. The positive control used for the PCR-based diagnostic assay was DNA extracted from a known culture of *A. fragariae* using the Dneasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA). The negative control for the PCR-based diagnostic assay was molecular grade dH_2O. Products of each PCR reaction were analyzed by gel electrophoresis through a 1.5% agarose gel buffered in 1.0X TBE, which contained ethidium bromide at a final concentration of 0.15 $\mu g/ml^{-1}$ for 60 minutes at 100 V. The gel was visualized under UV light.

Distribution of Foliar Nematodes Within The Plant Architecture

A block of *Verbena* 'Snowflurry' plants was selected for sampling from a commercial nursery because the plants were highly symptomatic of foliar nematode infection and would provide multiple replicates of infected plants to determine the spatial distribution of nematodes throughout the plant. Twenty-nine *Verbena* 'Snowflurry' plants were arbitrarily sampled from a 8.98 square meter nursery block containing 58 plants. Plants were removed from the overhead irrigation of the nursery to drip irrigation in a university greenhouse to prevent further spread of the nematode prior to processing. The complete shoot of each *Verbena* plant was removed to process for foliar nematode infection. One fully expanded leaf was collected at each internode beginning at the base of the plant (internode 1) and proceeding to the apex (internode 8). Each leaf was processed as one individual sample. Six 6mm leaf disks were taken from each leaf. Three of the leaf disks were used in the foliar

72

nematode water incubation extraction assay and the remaining 3 leaf disks were used for DNA extraction. The resulting total DNA was used as template for the AfragF1/AfragR1 PCR-based diagnostic assay described above. The results for each diagnostic assay were recorded and the number of positive results for each internode was tallied. A single foliar nematode identified from water extraction or the presence of the 169-bp PCR product was considered as "positive" detection. Foliar nematode detection by the water extraction assay was compared to the number of foliar nematode-positive samples found using the PCR-based diagnostic assay to determine if a difference exists between the sensitivity of the assays.

Distribution of Foliar Nematodes Within Blocks of Ornamental Nursery Plants

Anemone **Sampling**

A 21.68 sq. meter commercial nursery block that contained 140 *Anemone* plants of various species was sampled at two six-week intervals. Only one plant within the block was originally found to be symptomatic for foliar nematode infection, however, the block was overhead-irrigated, making an ideal atmosphere for foliar nematode spread. The block of *Anenome* plants was sampled to determine an effective sampling unit and to determine which assay was the most sensitive in detecting the presence of foliar nematodes. A leaf was excised from the top third, middle third, and the bottom third of each of 73 *Anemone* plants chosen arbitrarily within the block. The three leaves were compiled to provide one sample per plant. Each collected leaf was examined for symptomatic tissue and the results were recorded. Then, two leaf disks were collected from each leaf. One of the leaf disks per leaf was used in the water incubation extraction assay as described above and the remaining leaf disks were used for the AfragF1/AfragR1 PCR-based diagnostic assay as described above.

The *Anenome* whole leaf tissue remaining after the leaf disks were removed was thinly sliced into small pieces and used in the water extraction assay to compare to the leaf disk assay. Upon returning for the second sampling date, many of the plants had been sold. Therefore, the remaining plants 91 plants were sampled, using the same 3-leaf sampling protocol and detection assays as that of the first sampling date. The nursery owner also treated the remaining plants in the block with the miticide, chlorfenapyr (Pylon), which has been shown to have some effect on the population of foliar nematodes but has not been found to completely eradicate the nematodes from the foliage. Results from each *Anenome* sampling date for the water extraction assay were counted and compared to the positive results from the PCR-based diagnostic assay.

Disporum, *Oxalis*, and *Mildellia* sampling

The remaining three plant species, *D. smilacinum*, *O. regnellii*, and *M. nitidula*, were all located in a single commercial nursery, but in different greenhouses within the nursery. These nursery blocks were sampled because at least one plant in each block was found to be symptomatic for foliar nematode infection. This nursery also used overhead irrigation, making an ideal atmosphere for the spread of foliar nematodes. Two different nursery blocks of *Disporum smilacinum* 'Aureovariegata' plants were sampled, a larger 5.57 m^2 area containing 36 plants and a smaller 2.79 m^2 area containing 18 plants. The two plots were created by removing all 18 symptomatic plants from the nursery block and placed in a quarantine section of the nursery where the nematode spread could be controlled. The remaining 36 plants were then sampled and left in the original location. The second

sampling unit was sampled was a 5.57 m^2 area containing 36 *Oxalis regnelleii* plants. The third sample unit sampled was a 3.72 m^2 area containing 24 *Mildella nitidula* plants.

Plant sampling for foliar nematode detection within each block of plants was conducted in four, two-week intervals. All plants were initially numbered in chronological order beginning with the left side of the block and proceeding up and down the rows until the last plant was numbered. One leaf each was removed and combined from the top third, the middle third, and the bottom third of individual *D. smilacinum* and *O. regnellii* plants at each sampling. One frond on each *M. nitidula* plant was removed from outside, middle, and inside concentric circles. The three fronds were combined so there was one sample per *M. nitidula* plant on each sampling date. Leaf samples were either immediately processed for foliar nematode detection or placed in a 4º C refrigerator to be processed within 24 hours. The same plants were sampled on each sampling date to assess the temporal progression of foliar nematode infection in each plant species. Each of the samples was split and evaluated using both the traditional foliar nematode water extraction assay and the AfragF1/AfragR1 PCR-detection assay.

Two 6-mm leaf disks were removed from each combined leaf sample at each sampling date. Three leaf disks representing each *D. smilacinum* and *O. regnellii* leaf in the combined sample were used for the water incubation extraction assay and a second three leaf disk sample was used in the PCR-based diagnostic assay. To process the *M. nitidula* frond samples, two leaflets from each frond were removed; one of the leaflets from each frond, for a total of three leaflets was used for the water incubation extraction assay and the remaining three leaflets were used for the PCR-based diagnostic assay. The whole leaf tissue remaining after the leaf disks were removed from each sample was thinly sliced in to small pieces and

used in the water extraction assay to compare to the leaf disk assay. A single foliar nematode identified from water extraction or the presence of the 169-bp PCR product was considered as "positive" detection. In addition, the number of foliar nematodes was counted in each leaf disk and whole leaf water extraction sample for a quantitative assessment of foliar nematode infection of each replicate plant.

Results were analyzed to determine which detection assay was the most efficient, the proportions of positive results for each host species, the spatial distribution of the nematodes throughout the sampling block, and an appropriate sample size. The percent of positive samples were examined by determining the number of positive samples as related to total plants sampled. From these results the proportion of positive results by each detection method was determined. A paired t-test was performed on the raw data to determine any significant difference between the two foliar nematode detection assays. Quantitative assessment of foliar nematode infection derived in the water extraction method was also used to determine optimum sample size using Taylor's Power Law (Taylor, 1961). Taylor's Power Law is a function that examines the relationship between the variance and mean (Davis, 1994; Taylor, 1961). To perform Taylor's Power Law, the logarithm of the mean calculated by taking the average number of nematodes collected using the water extraction assay during each sampling date was plotted against the logarithm of the variance of these data. The slope (b) of the line was indicative of the degree of aggregation of the nematodes within the sampling block (Davis, 1994; Taylor, 1961). Taylor's Power Law states that if a population is random b=1, uniform b<1, and aggregated b>2 (Taylor, 1961). The Taylor's Power Law coefficients were subsequently used to determine the sampling size needed to detect the nematodes in a nursery block at a precision of p=0.25 (Wilson et al., 1983).

76

RESULTS

Detection Methods for Foliar Nematodes in Plant Samples

All leaves from 29 symptomatic *Verbena* 'Snowflurry' plants were assayed for the presence of foliar nematodes in split samples by conventional water extraction and *A. fragariae*-specific PCR. Beginning with the first internode at the bottom of the plant, 16 samples were positive for at least one foliar nematode by water extraction, while 11 samples were *A. fragariae*-PCR assay positive (Figure 1). At the second internode, 14 samples were water extraction positive, in contrast 12 samples positive by PCR. Twelve samples were water extraction positive and 11 samples were PCR assay positive at the third internode. At the fourth internode, 7 samples were water extraction positive, while 8 samples were PCR assay positive. At the fifth internode, 6 samples were water extraction positive, while 2 were PCR assay positive. At the sixth internode, 1 sample was water extraction positive and 1 sample was PCR-assay positive. At the seventh internode 2 samples were water extraction positive and one sample was PCR assay positive. At internode 8, no samples were found to be water extraction positive while one sample was PCR positive.

Distribution of Foliar Nematodes Within Blocks of Ornamental Nursery Plants

Anemone Sampling

Only one plant among the seventy-three plants initially sampled in the block of 140 *Anenome* plants was symptomatic for foliar nematode infection at the first sampling date, however, foliar nematodes were not detected in the leaf tissue collected from this plant using either detection method. Only three *Anemone* plants in this first sampling date were positive for foliar nematodes using the water extraction method, while the PCR-based diagnostic

assay detected foliar nematodes in 51 of the 73 plants in the initial sampling (data not shown). All three samples that were positive using the water extraction assay were also positive using the PCR-based diagnostic assay. Of the ninety-one symptomatic *Anemone* plants that remained in the block six weeks later, only one was positive for foliar nematodes using the water extraction assay, but 28 were found to be positive for foliar nematode infection using the PCR-based diagnostic assay. Again, the sample that tested positive using the water extraction assay was also positive using the PCR-based diagnostic assay.

Disporum, *Oxalis*, and *Mildellia* Sampling

Disporum smilacinum

In the initial sampling of 36 plants in the *D. smilacinum* nursery block, foliar nematodes were detected in 52.7% of the leaf disk samples by the PCR assay as compared to 7.0% from water extraction of the remaining leaf disk samples (Figure 2, Plate A). Foliar nematodes were detected in none of the whole leaf samples by the water extraction assay in the initial nursery sampling. From leaf disk samples of the same 36 *D. smilacinum* plants in the nursery block two weeks later, 77.7% were positive for foliar nematode infection using the PCR detection assay as compared to 22.2% from water extraction assay of the leaf disk sample (Figure 2, Plate B). 22.2% were positive for foliar nematode infection using the water extraction on the whole leaf disk sample on the second sampling date. From leaf disk samples of the same 36 *D. smilacinum* plants in the nursery block on the third sampling date, 54.3% of the leaf disk samples were positive for foliar nematode infection using the PCR-detection assay compared to 22.9% detected by water extraction of the leaf disks (Figure 2, Plate C). 22.9% of the whole leaf samples were positive using the water extraction method. At the fourth sampling date, 30.5% of the leaf disk samples were positive for foliar nematode

infection using the PCR detection assay compared to 33.3% positive in the leaf disk sample

by water extraction. (Figure 2, Plate D). 30.5% of the whole leaf samples were positive for

foliar nematode infection using the water extraction method in the final sampling. The results

from the first three sampling dates using both the PCR-detection assay and the water

extraction assay remained relatively constant, with a peak detection of foliar nematodes

during the second sampling date (Figure 2). The number of nematodes detected using both

the PCR-detection assay and the water extraction assay, however, substantially decreased by

the fourth sampling date. The detection of plant symptoms of foliar nematode infection

gradually increased throughout the sampling period to a maximum of about one-third of the

plants showing disease symptoms by the fourth sampling date. Over the four sampling dates,

foliar nematodes were detected in a significantly (p=0.0359) higher percentage of *D.*

smilacinum nursery samples using the PCR detection versus the water extraction assay of

split leaf disk samples.

In the initial sampling of 18 plants in the second, quarantined *D. smilacinum* nursery

block, foliar nematodes were detected in 94.4% of the leaf disk samples by the PCR assay as

compared to 55.6% from water extraction of the remaining leaf disk samples (Figure 3, Plate

A). Foliar nematodes were detected in 88.8% of the whole leaf samples by the water

extraction assay in the initial nursery sampling. From leaf disk samples of the same 18 *D.*

smilacinum plants in the nursery block two weeks later, 72.2% were positive for foliar

nematode infection using the PCR detection assay as compared to 55.5% from water

extraction assay of the leaf disk sample (Figure 3, Plate B). 50.0% were positive for foliar

nematode infection using the water extraction on the whole leaf sample. From leaf disk

samples of the same 18 *D. smilacinum* plants in the nursery block on the third sampling date,

72.2% of the leaf disk samples were positive for foliar nematode infection using the PCR-detection assay compared to 66.7% detected by water extraction of the leaf disks (Figure 3, Plate C). 72.2% of the whole leaf samples were positive using the water extraction method. At the fourth sampling date, 88.9% of the leaf disk samples were positive for foliar nematode infection using the PCR detection method compared to 77.8% of the whole leaf sample (Figure 3, Plate D). 55.6% of the whole leaf samples were positive using the water extraction method. Two-thirds of the plants displayed disease symptoms of foliar nematode infection by the second sampling date and this level of symptom observation remained through the fourth sampling date. Over the four sampling dates, the difference between the two foliar nematode detection assays in the quarantined block of *D. smilacinum* plants was not statistically (p=0.0603) significant.

Taylor's Power Law (Taylor, 1961) was used to determine the distribution of foliar nematode infestation within the combined blocks of 54 *D. smilacinum* plants. Foliar nematode counts from water-extracted leaf disk and whole leaf samples from each sampling date provided the raw data for the distribution analyses. Plotting Log_{10}mean x Log_{10}variance from the leaf disk, whole leaf, and combined assay of both leaf disk and whole leaf data, indicated that foliar nematode distribution was aggregated (b-values > 2.0) in the *D. smilacinum* blocks. The sampling sizes for each of the assays were found using an algorithm derived from Taylor's Power Law (Wilson et al., 1983). The predicted optimal sampling size varied from 22 to 27 based on the degree of aggregation as determined by different leaf samples and the slope of the line of best fit at a precision of p=0.25. At the calculated sampling size and distribution, foliar nematodes should be detected 75% of the time using the water extraction assay.

80

Mildella nitidula

In the initial sampling of 24 plants in the *M. nitidula* nursery block, foliar nematodes were detected in 45.8% of the leaf disk samples by the PCR assay as compared to 50.0% from water extraction of the remaining leaf disk samples (Figure 5, Plate A). Foliar nematodes were detected in 45.8% of the whole leaf samples by the water extraction assay in the initial nursery sampling. From leaf disk samples of the same 24 *M. nitidula* plants in the nursery block two weeks later, 50.0% were positive for foliar nematode infection using the PCR detection assay as compared to 79.2% from water extraction assay of the leaf disk sample (Figure 5, Plate B). 50.0% were positive for foliar nematode infection using the water extraction in the second whole leaf disk sampling. From leaf disk samples of the same 24 *M. nitidula* plants in the nursery block on the third sampling date, 79.2% of the leaf disk samples were positive for foliar nematode infection using the PCR-detection assay compared to 100.0% detected by water extraction of the leaf disks (Figure 5, Plate C). 82.6% of the whole leaf samples were positive suing the water extraction method in the third sampling. At the fourth sampling date, 75.0% of the leaf disk samples were positive for foliar nematode infection using the PCR detection method compared to 54.2% of the whole leaf sample (Figure 5, Plate D).

Essentially all plants in the *M. nitidula* block displayed disease symptoms of foliar nematode infection from the initial sampling through the fourth sampling date. Over all four sampling dates, the difference between the two foliar nematode detection assays in *M. nitidula* was not statistically (p=0.7048) significant.

A Log$_{10}$ mean-variance graph with a line of best fit using Taylor's Power Law was developed for the quantitative data of nematode counts from water extraction of foliar nematodes from leaf disks and whole leaves of *M. nitidula* (Figure 6). The b-values for the leaf disk water extraction assay and the analysis of combining the water extraction assay were found to be 1.56 and 1.54, respectively, meaning the foliar nematode distribution among *M. nitidula* was less aggregated throughout the sampling plot than observed in the *D. smilacinum* block. However, log mean vs. log variance from the whole leaf assay alone suggested the nematodes were more uniformly distributed throughout the sampling plot. A sampling size for each of the water extraction assay was found. The sample size at a precision of p=0.25 for the leaf disk assay and the combination of the two assays was 41.38 and 36.27 respectively, due to the predicted degree of aggregation and the slope of line of best fit (Figures 6). The sampling size at a precision of p=0.25 for the whole leaf assay was 21.76 samples since the population was predicted as more uniformly distributed (Figures 6).

Oxalis regnellii

In the initial sampling of 36 plants in the *O. regnellii* nursery block, foliar nematodes were detected in 97.2% of the leaf disk samples by the PCR assay as compared to 94.4% from water extraction of the remaining leaf disk samples (Figure 7, Plate A). Foliar nematodes were detected in 94.4% of the whole leaf samples by the water extraction assay in the initial nursery sampling. From leaf disk samples of the same 36 *O. regnellii* plants in the nursery block two weeks later, 83.3% were positive for foliar nematode infection using the PCR detection assay as compared to 22.2% from water extraction assay of the leaf disk sample (Figure 7, Plate B). 55.6% were positive for foliar nematode infection using the water extraction in the second whole leaf disk sampling. From leaf disk samples of the same

82

36 *O. regnellii* plants in the nursery block on the third sampling date, 52.7% of the leaf disk samples were positive for foliar nematode infection using the PCR-detection assay compared to 22.2% detected by water extraction of the leaf disks (Figure 7, Plate C). 38.9% of the whole leaf samples were positive in the third sampling date using the water extraction method. At the fourth sampling date, 71.4% of the leaf disk samples were positive for foliar nematode infection using the PCR detection method compared to 34.3% of the whole leaf sample (Figure 7, Plate D). 31.4% of the whole leaf samples were positive using the water extraction method in the final sampling. Symptoms of foliar nematode infection were observed on about 50% of *O. regnellii* plants at the initial sampling date, on less than 25% of the plants at the second and third sampling dates, and symptoms were observed on about 75% of *O. regnellii* plants at the fourth sampling date. Over all four sampling dates, foliar nematodes were detected in a significantly (p=0.0106) higher percentage of *O. regnellii* nursery samples using the PCR detection assay of leaf disk samples than the water extraction assay of the leaf disk samples on foliar nematodes.

Log$_{10}$ mean-variance graph with a line of best fit using Taylor's Power Law was developed for the quantitative data of nematode counts from water extraction of foliar nematodes from *O. regnellii* (Figure 8). Analyses of counts of foliar nematodes from water extraction of leaf disks, whole leaves, and both tissues combined provided b-values above 2.0, indicating the nematode distribution in the block to be aggregated. The b-value from the water extraction assay using only leaf disks was 2.57, predicting an large optimal sampling size at p=0.25 precision of 38 plants. However, for the whole leaf assay and the combination of the two the b-values are closer to 2.0, providing .25 precision predicted sampling sizes of 20 and 24 plants, respectively.

DISCUSSION

The objective of these studies was to assess the distribution of foliar nematode infection within plant architecture and the distribution of foliar nematode infestation among blocks of several different ornamental plant species grown in commercial nurseries. The relative efficiency of nematode detection by the conventional water extraction assay (Jagdale and Grewal, 2002) versus a new PCR-based foliar nematode (*A. fragariae*) detection assay (McCuiston, 2007) was also assessed among the same samples. An optimal sampling size for each plant block at p=0.25 precision was also calculated based upon the distribution of the nematodes as derived by Taylor's Power Law (Taylor, 1961).

The *Verbena* 'Snowflurry' plants were highly symptomatic for foliar nematode infection with symptomatic leaves present throughout each plant sampled. The symptoms corresponded closely to the number of positive samples found using both the detection methods. Nematodes were found throughout the infected *Verbena* plant, with the highest concentration of nematodes at the bottom third of the plant. This was consistent with the observed movement of foliar nematodes in herbaceous perennials as they break dormancy in the spring, where *A. fragariae* migrate from the soil or plant crown up the plant stems infecting the lower leaves first and continue to migrate up as the season continues (Decker, 1972; Jagdale and Grewal, 2006). Foliar nematode distribution may vary, however, based on the architecture differences among plant species. Foliar nematode distribution within a plant can be further impacted by splash dispersal or introduction by mechanical means from plant to plant in water or horticultural tools (Daughtrey et al., 1995). To account for these confounding factors, a standardized protocol was used here where the plant to be sampled

84

was divided into three equal sections to fully sample a plant regardless of plant architecture or how the plant was infected by foliar nematodes.

In most commercial plant nursery and greenhouse operations, plants are not routinely tested for the presence of foliar nematodes unless the plant is symptomatic. Assessment of foliar nematode infection of symptomatic plants is typically to confirm if the plant is, in fact, infected so the plant can be removed or another control measure can be employed. However, the surrounding plants are not sampled to determine if the nematodes have spread. The two detection assays here were used to determine which of the assays is the most sensitive. The PCR-based detection assay was able to detect a statistically higher proportion of foliar nematode infection for both the *O. regnellii* and the *D. smilacinum* blocks plants. These plants were relatively asymptomatic until the last sampling date. The observed decrease in the ability of the PCR-detection assay to consistently detect nematodes in *D. smilacinum* over the four sampling dates was curious, however, and could have be due to several factors. First, the *D. smilacinum* plants were located in a greenhouse covered with shade cloth, which is the optimal growing conditions for this species of plant. However, the temperature inside the greenhouse increased over the four sampling dates, exceeding the optimal temperature for *A. fragariae.* This may have caused a decrease in nematode reproduction with a concomitant increase in plant symptom development under heat stress. Second, the *D. smilacinum* sampling was destructive -- as the sampling progressed any infected leaves may have been removed from lightly-infected plants. It is believed that with the increase in temperature causing a decrease in the reproduction of the nematode as well as the destructive sampling, the data found using the PCR-detection assay data from the first three sampling dates were not false positives. The *O. regnellii* plants were located in a greenhouse covered with shade

85

cloth, which is the optimal growing condition for this species of plant. However, the

temperature inside the greenhouse increased over the four sampling dates, exceeding the

optimal temperature for *A. fragariae* reproduction. The *O. regnellii* were symptomatic

throughout the sampling dates, therefore like the *M. nitidula* there was not a decrease in

detection using either the water extraction assay or the PCR-based assay. This could be

attributed to the abundance of nematodes in the symptomatic plants that were even though

there may have been a decrease in reproduction due to the increase in temperature the

nematodes were able to spread and infect other plant species.

The PCR-based assay also detected foliar nematode infection in a greater number of

plants than the water extraction assay in the nursery block containing the *Anemone* spp.

Foliar nematode detection by PCR greatly decreased in the Anemone block after six weeks,

however, following application of the miticide chlorfenapyr. Chlorfenapyr has been shown to

have some effect on the population of foliar nematodes but has not been found to completely

eradicate the nematodes from the foliage (Williams-Woodward, 2003). The PCR assay did

not detect a significantly greater number of positive samples than the water extraction assay,

however, in the *M. nitidula* and *D. smilacinum* located in the quarantine area of the nursery.

The plants located in both of these sampling plots were almost all showing symptoms. The

plants do not begin to show symptoms until the leaf is heavily infected. The water extraction

assay relies on the ability of the water to migrate out into the surrounding water and the

expertise of someone trained to identify foliar nematodes based on morphology. Therefore,

at low populations the possibility of error using the water extraction assay increases. A

comparison of nematode detection using the water extraction assay for both leaf disks and

whole leaves indicated comparable detection of nematode infection using either tissue

86

sample size. The PCR assay has been found to accurately detect one nematode in a sampling unit of 3 leaf disks (McCuiston, 2007). The assay has also been found to accurately detect 1,000 nematodes in three leaf disks. It can be concluded that in order to effectively detect the presence of foliar nematodes in infected asymptomatic tissue the PCR-based diagnostic assay is more sensitive, but once the plant is symptomatic, either foliar nematode detection assay is effective.

Most plant-parasitic nematode populations in soil have been shown to have an aggregated distribution (Anscombe, 1950; Boag, 1984; Goodell and Ferris, 1980; Perry, 1983), but data on foliar nematode distribution in ornamental plant nurseries has been lacking. Since the water extraction assays presented actual counts of nematode populations within plants, the data could be used to assess foliar nematode distribution within blocks of plants using Taylor's Power Law (Taylor, 1961). The nematodes within *D. smilacinum* and *O. regnellii* were found to be aggregated within the nursery block while the *M. nitidula* were less aggregated, approaching a random distribution. The majority of the plants sampled in both *D. smacilium* and *O. regnellii* were asymptomatic but as the nematodes spread throughout the block and more of the plants were positive using the water extraction assay, the less aggregated the nematodes are within the nursery block as predicted.

The Taylor's Power coefficients (a=slope and b=intercept) were subsequently used to determine optimal sampling size based on the algorithm developed by Wilson et al. (1983). The sampling size is based on a precision of p=0.25 meaning that the nematodes if present will be located 75% of the time, which is appropriate for pest management purposes. Based upon the foliar nematode distribution calculated, the predicted optimal sampling size for the *D. smilacinum* block ranged from 21 to 27, the sampling size for *O. regnellii* ranged from 21

to 38, and *M. nitidula* sample size ranged from 21 to 41. Sampling size is dependent upon environmental conditions, the size of the sampling area, the density of the host plants, the number of plants in the sampling area, the type of irrigation used, and the overall condition of the sampling area. The sample size and sampling protocol need to be validated using the same conditions as those where the sampling sizes was determined before the protocol could be recommended for use to detect propagative stock, incoming, and existing plants for foliar nematode infection and management. Given the significant increase in sensitivity of the PCR-based detection assay as compared to water extraction, it is likely that the sample size could be further reduced using PCR-detection of foliar nematodes. Real-time PCR may be used to quantify foliar nematode infection levels in samples to provide the raw data to derive nematode distribution patterns and optimal sample sizes using Taylor's Power Law.

The integration of a sensitive and objective assay to detect foliar nematode infection in asymptomatic ornamental plant species with knowledge of foliar nematode distribution in commercial nursery operations presents a powerful tool to make nematode management decisions. Early detection of foliar nematodes will help to prevent the propagation and introduction of foliar nematode-infested plant material into a nursery, reduce subsequent spread of foliar nematodes throughout the facility, and promote more efficient management of foliar nematode infestation in the ornamental plant industry.

LITERATURE CITED

Abd-Elgawad, M.M. and Hasabo, S.A. 1995. Spatial distribution of the phytonematode commonly in Egyptian erseem Clover (*Trifolium alexandrinum L.*) fields. Fundamentals of Applied Nematology. 18 (4): 329-334.

Anscombe, F.J. 1950. Soil sampling for potato root eelworm cysts. Annals of applied Biology. 37: 286-295.

Boag, B. and Topham, P.B. 1984. Aggregation of Plant Parasitic Nematodes and Taylor's Power Law. Nematologica. 30:348-357.

Buckley, R.J. and Gould, A.B. 2003. Foliar nematodes in ornamental plants. Plant Disease Control Fact Sheet. Rutgers Co-operative Extension, The State University of New Jersey. http://www.rce.rutgers.edu/pubs/pdfs/fs878.pdf.

Davis, P.M. 1994. Handbook of Sampling Methods for Arthropods in Agriculture. Pedigo, L.P. and Buntin, G.D. eds. CRC Press. Boca Raton, FL. 33-54.

Daughtrey, M.L., Wick, R.L., and Peterson, J.L. Compendium of Flowering Potted Plant Diseases. 1995. APS Press, St. Paul. MN, p.75-76.

Decker, H., 1989. Leaf- Parasitic Nematodes. In *Plant Nematodes and Their Control*. Sveshnikova, N. M. Ed. Kinderbrook, NY. E.J. Brill Publishing Company, 354-358.

De Maeseneer, J. 1964. Leaf- Browning of *Ficus* spp., new host plants of *Aphelenchoides fragariae* (Ritzema bos). *Nematologica*. 10:403-408.

Duncan, L.W., El-Morshedy, M.M. and McSorley, R. 1994. Sampling citrus fibrous roots of *Tylenchulus semipenetrans*. Journal of Nematology. 26(4): 442-451.

Dunn, R. A. Foliar Nematodes in Nursery Crops. 2005. University of Florida: IFAS

Extension. URL:

http://ipm.ifas.ufl.edu/applying/tactics/pesticides/foliar_nematodes_nursery_crops.ht

m

Goodell, P. and Derris, H. 1980. Plant Parasitic nematode distribution in an alfalfa field.

Journal of Nematology. 12:136-143.

Jagdale, G.B., and Grewal, P.S. 2002. Identification of alternatives for the management of

foliar nematodes in floriculture. *Pest Management Science*. 58: 451-458.

Jagdale, G.B., and Grewal, P.S. 2004. Effectiveness of a Hot Water Drench for the Control

of Foliar Nematodes Aphelenchoides fragariae in Floriculture. *Journal of

Nemataology*. 36(1): 49-53.

Jadgale, G.B. and Grewal, P.S. 2006. Infection Behavior and Overwintering Survival of

Foliar Nematodes *Aphelenchoides fragariae*, on Hosta. *Journal of Nematology*. 38(1):

130-136.

Johnson, A.W. and Gill, D.L. 1975. Chemical control of foliar nematodes (*Aphelenchoides

fragariae*) on 'Fluffy Ruffles' Fern. *Plant Disease Reporter*. 59(9) 772-774.

Kashaija, I.N., McIntyre, B.D., Ssali, H. and Kizito, F. 2004. Spatial distribution of roots,

nematode populations and root necrosis in highland banana in Uganda. Nematology.

6(1): 7-12.

La Mondia, J.A., 1999. Efficacy of Insecticides for Control of *Aphelenchoides fragariae* and

Ditylenchus dipsaci in Flowering Perennial Ornamentals. *Journal of Nematology*.

31(4S): 644-649.

Mac Lachlan, J.B. and Duggan, J.J. 1979. An effective method for hot-water treatment and propagation of strawberry runners. Irish Journal of Agriculture Research 18:301-304.

McCuiston, J.L. 2007. Development of a PCR-based Diagnostic Assay and Sampling Protocol for Detection of *Aphelenchoides fragariae* in Ornamental Nursery Crops. MS Thesis, North Carolina State University.

Mor, M. and Spiegel, Y. 1993. *Ruscus hypophyllum*: A New Host for *Aphelenchoides fragariae. Journal of Nematology*. 25(2):312-313.

Perry, J.N. 1983. Effects of spatial heterogeneity of Jones's Model of Cyst Nematode Population Dynamics and Crop Root Damage. Journal of Applied Ecology. 20: 849-856.

Sanwal, K.C. 1959. A simple method of rearing pure populations of the foliar nematode, *Aphelenchoides ritzemabosi*, in the laboratory. *Canadian Journal of Zoology*. 37:707-711.

Schulze, L. 2003. Federally registered restricted use pesticides. Clemson University Pesticide information Program. 1-16, http://entweb.clemson.edu/pesticid /document/fedrup.htm.

Singh, R. S. and Sitaramaiah, K. 1994. Plant Pathogens: The Plant Parasitic Nematodes. International Science Publisher, New York, NY. 261-289.

Southwood, T.R.E. and Henderson, P.A. 2000. Ecological methods Third Edition. Blackwell Science Led. Malden, MA. 15-26.

Taylor, L. R. 1961. Aggregation, Variance, and the Mean. Nature. 189: 732-735.

Thorne, G. 1961. Principles of Nematology. McGraw- Hill Book Company, Inc. New York, NY. 410-427.

Vovlas, N., Minuto, A., Garibaldi, A., Troccoli, A., and Lamberti, F. 2005. Identification and histopathology of the foliar nematode *Aphelenchoides ritzemabosi* (Nematoda: Aphelenchoididae) on basil in Italy. *Nematology*. 7(2): 301-308.

Wallace, H.R. 1959. Movement of eelworms V. Observations of *Aphelenchoides ritzemabosi* on florist chrysanthemum. *Annuals of Applied Biology*. 47: 350-360.

Williams-Woodward, J.L. 2003. Combating foliar nematodes on herbaceous garden plants. URL: http://www.growit.com/Features/ghbiz504.htm

Wilson, L.T., Gonzalez, D., Leigh, T.F., Maggi, V., Foristiere, C., and Goodell, P. 1983. Within-Plant Distribution of Spider Mites (Acari: Tetranychidae) on Cotton: A Developing Implementable Monitoring Program. Environmental Entomology. 12: 128-134.
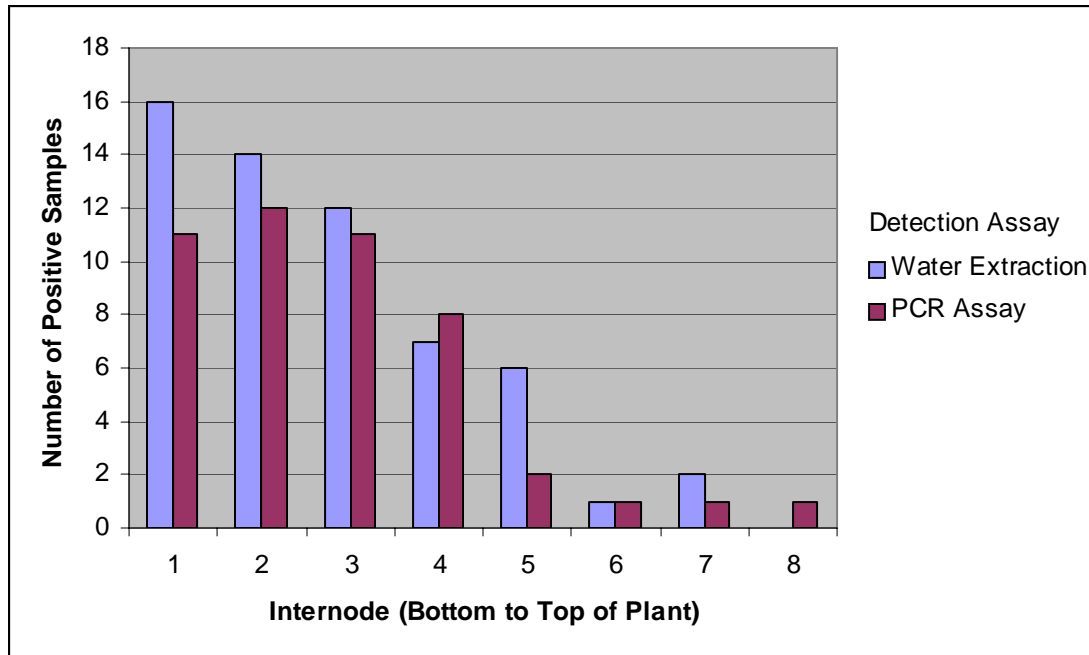
Figure 1. Distribution of foliar nematode (*Aphelenchoides fragariae*) infection (positive samples) within the architecture of Verbena 'Snowflurry' plants sampled from each internode beginning at the bottom of the plant with internode 1 and proceeding to the top with internode 8. Foliar nematode detection assays were conducted in split samples using a water extraction method (Vovlas et al., 2004; Jagdale and Grewal, 2002) and PCR-based detection (McCuiston, 2007) from 29 Verbena plants sampled.

Plate A: Sampling Date 5/31/2006
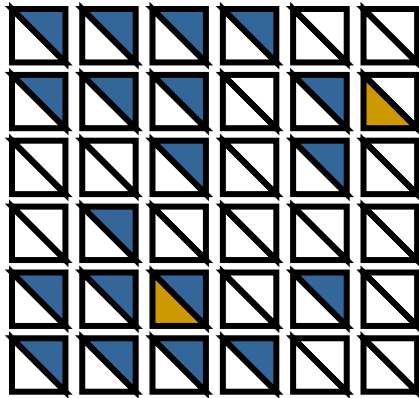
Plate B: Sampling Date 6/15/2006

Plate C: Sampling Date 6/30/2006

Plate D: Sampling Date 7/14/2006

PCR Both + Asymptomatic

H2O + Symptomatic
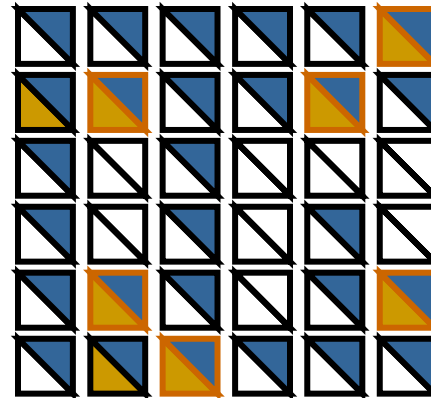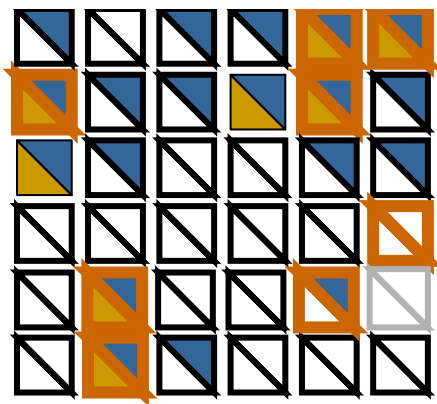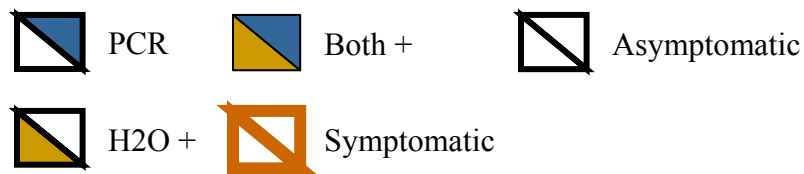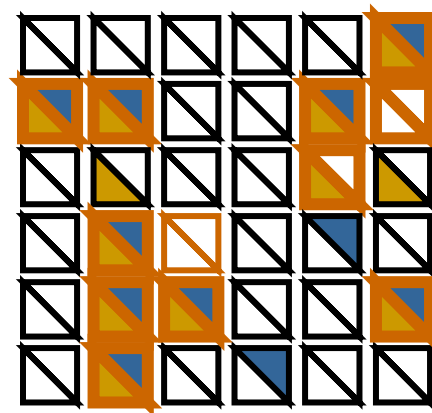
Figure 2. Detection of foliar nematode (*Aphelenchoides fragariae*) infection and symptoms in a block of 36 *Disporum smilacinum* 'Aureovariegata' plants over four sampling dates (Plates A-D). Foliar nematode detection assays were conducted in split leaf disk samples using a water extraction method (Vovlas et al., 2004; Jagdale and Grewal, 2002) and PCR-based detection (McCuiston, 2007) from all plants.
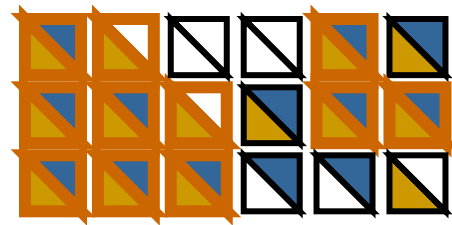
**Plate A: Sampling date 5/31/2006**
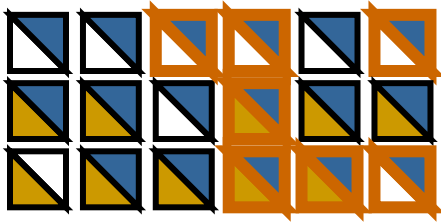
Plate B: Sampling Date 6/15/2006

Plate C: Sampling Date 6/30/2006

Plate D: Sampling Date 7/14/2006

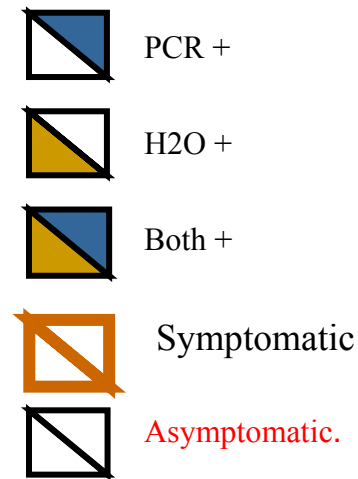PCR   Both +   Asymptomatic

H2O +   Symptomatic
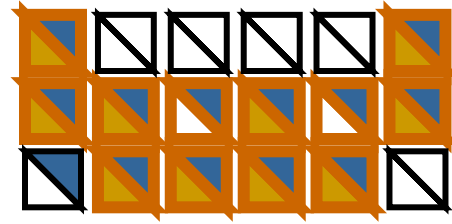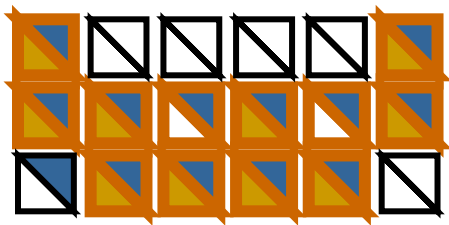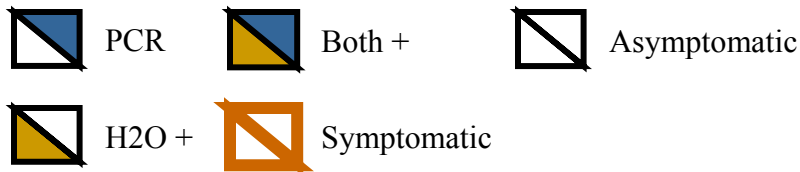
Figure 3. Detection of foliar nematode (*Aphelenchoides fragariae*) infection and symptoms in a second, quarantined block of 18 *Disporum smilacinum* 'Aureovariegata' plants over four sampling dates (Plates A-D). Foliar nematode detection assays were conducted in split leaf disk samples using a water extraction method (Vovlas et al., 2004; Jagdale and Grewal, 2002) and PCR-based detection (McCuiston, 2007) from all plants.
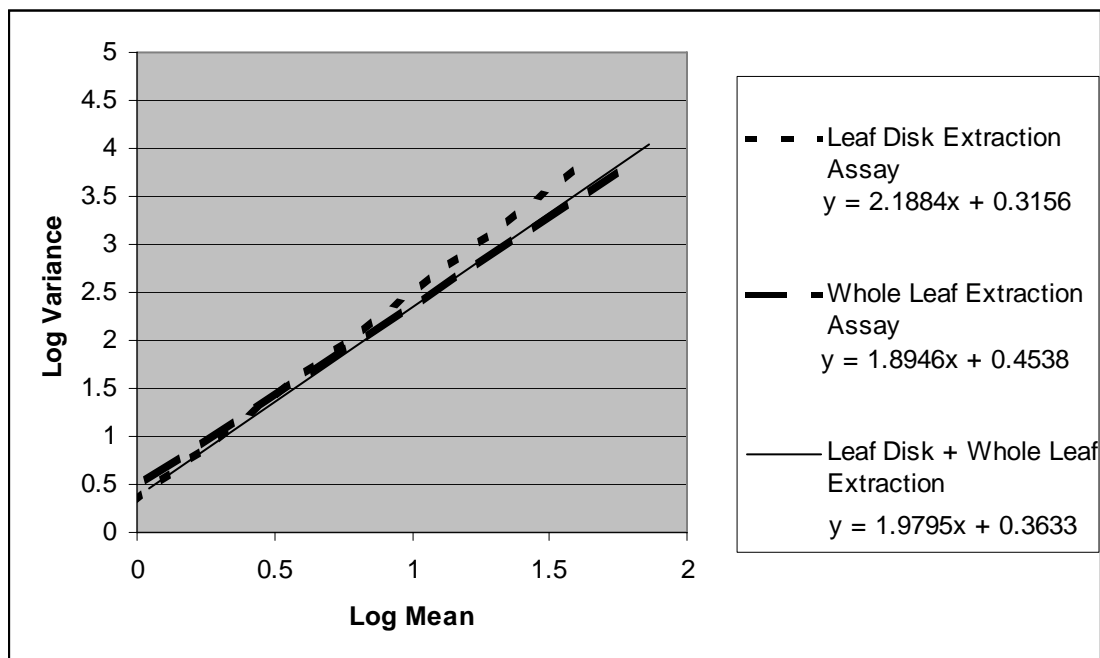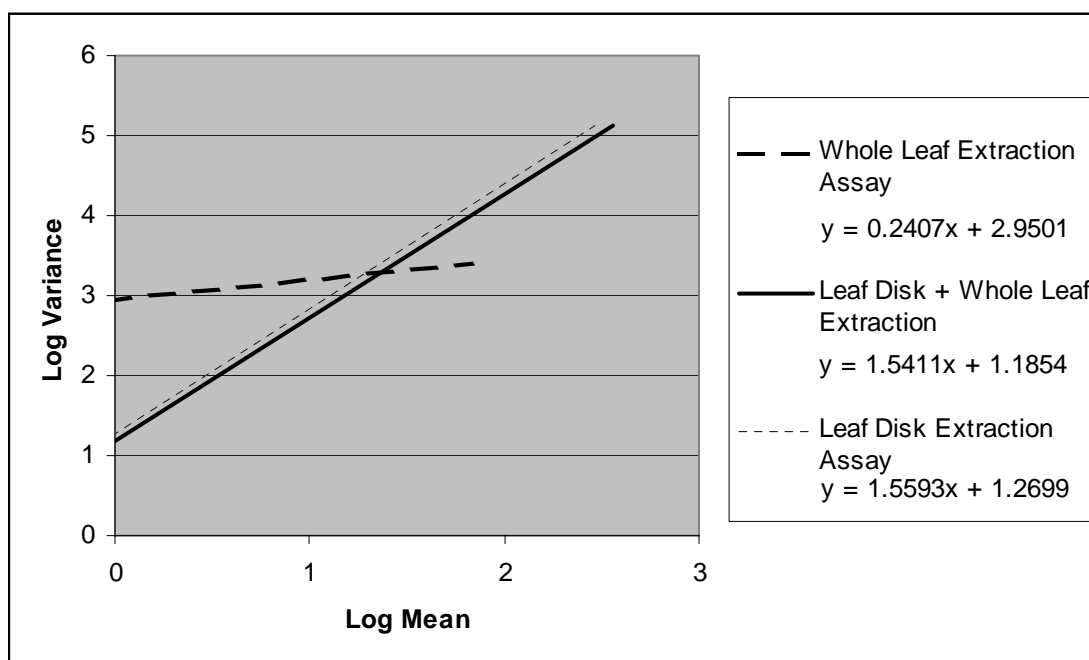
Figure 4. Use of Taylor's Power Law (Taylor, 1961) to determine the spatial distribution of *Aphelenchoides fragariae* infestation in a combination of two sampling blocks containing a total of 54 *Disporum smilacinum* 'Aureovariegata' plants. The mean and variance in number of nematodes collected using the water extraction assay for both the leaf-disk and the whole-leaf sampling units in each of four sampling dates were calculated. Line of best fit was determined for the Log_{10} of the mean and variance of replicate samples of foliar nematodes that were water-extracted and counted from leaf-disk and whole-leaf samples as two separate sampling units and one combined unit. Nematode distribution within the sample block is considered as aggregated if the slope (b-value) is greater than 2.00, random if the slope equals 1.00, and more uniform as the slope approaches 0.00.
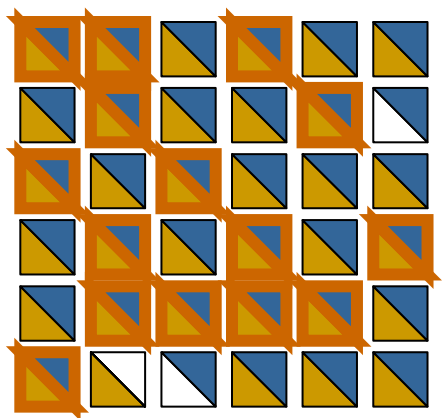
Plate A: Sampling date 5/31/2006

Plate B: Sampling Date 6/15/2006

Plate C: Sampling Date 6/30/2006

Plate D: Sampling Date 7/14/2006

PCR +

H2O +

Both +

Symptomatic

Asymptomatic.

Figure 5. Detection of foliar nematode (*Aphelenchoides fragariae*) infection and symptoms in a block of 24 *Mildella nitidula* plants over four sampling dates (Plates A-D). Foliar nematode detection assays were conducted in split leaf disk samples using a water extraction method (Vovlas et al., 2004; Jagdale and Grewal, 2002) and PCR-based detection (McCuiston, 2007) from all plants.
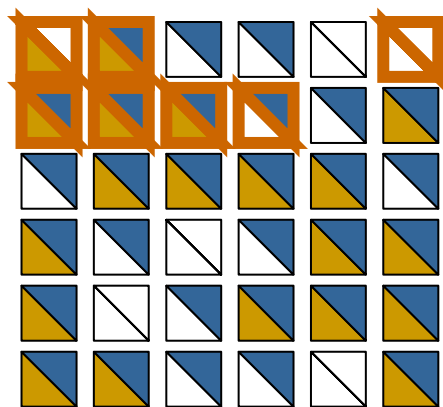
Figure 6. Use of Taylor's Power Law (Taylor, 1961) to determine the spatial distribution of *Aphelenchoides fragariae* infestation in a combination of two sampling blocks containing a total of 24 *Mildella nitidula* plants. The mean and variance in number of nematodes collected using the water extraction assay for both the leaf-disk and the whole-leaf sampling units in each of four sampling dates were calculated. Line of best fit was determined for the Log_{10} of the mean and variance of replicate samples of foliar nematodes that were water-extracted and counted from leaf-disk and whole-leaf samples as two separate sampling units and one combined unit. Nematode distribution within the sample block is considered as aggregated if the slope (b-value) is greater than 2.00, random if the slope equals 1.00, and more uniform as the slope approaches 0.00.
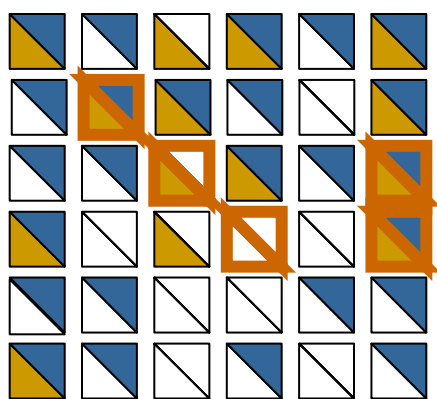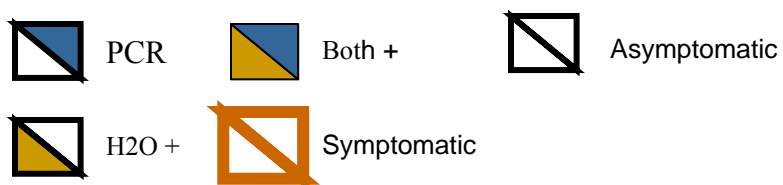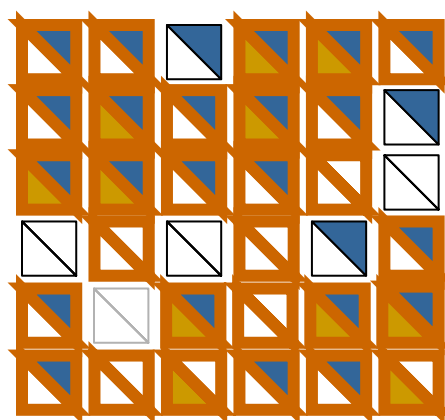
Plate A: Sampling Date 5/31/2006

Plate B: Sampling Date 6/15/2006

Plate C: Sampling Date 6/30/2006

Plate D: Sampling Date 7/14/2006

PCR

Both +

Asymptomatic

H2O +

Symptomatic

Figure 7. Detection of foliar nematode (*Aphelenchoides fragariae*) infection and symptoms in a block of 36 *Oxalis regnellii* plants over four sampling dates (Plates A-D). Foliar nematode detection assays were conducted in split leaf disk samples using a water extraction method (Vovlas et al., 2004; Jagdale and Grewal, 2002) and PCR-based detection (McCuiston, 2007) from all plants.
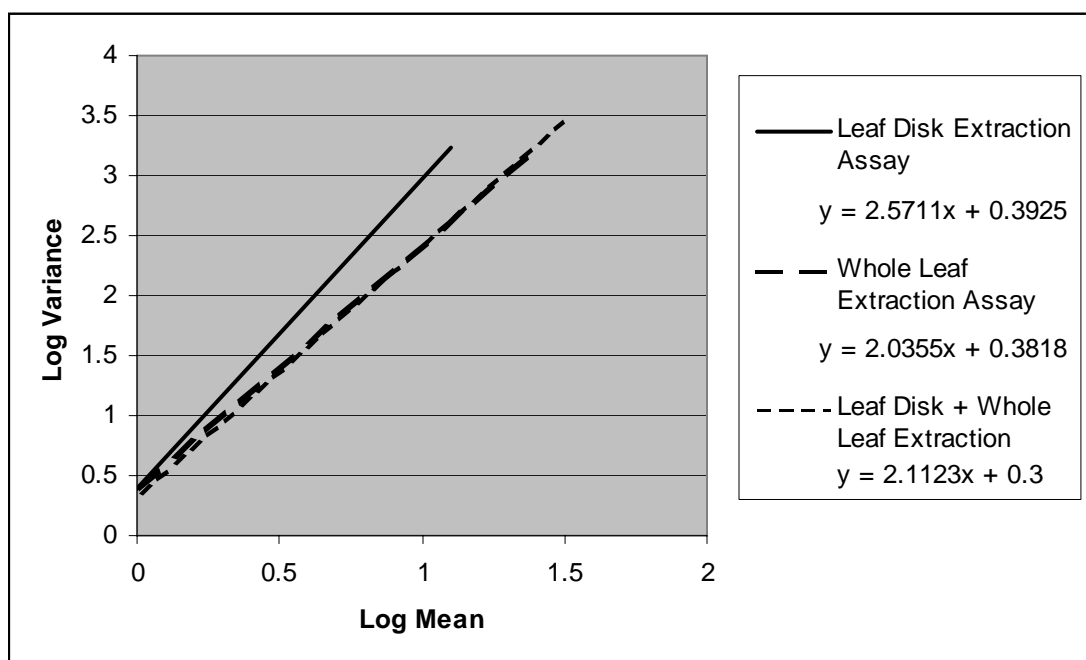
Figure 8. Use of Taylor's Power Law (Taylor, 1961) to determine the spatial distribution of *Aphelenchoides fragariae* infestation in a combination of two sampling blocks containing a total of 36 *Oxalis regnellii* plants. The mean and variance in number of nematodes collected using the water extraction assay for both the leaf-disk and the whole-leaf sampling units in each of four sampling dates were calculated. Line of best fit was determined for the Log_{10} of the mean and variance of replicate samples of foliar nematodes that were water-extracted and counted from leaf-disk and whole-leaf samples as two separate sampling units and one combined unit. Nematode distribution within the sample block is considered as aggregated if the slope (b-value) is greater than 2.00, random if the slope equals 1.00, and more uniform as the slope approaches 0.00.