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# THE FOLIAR NEMATODE APHELENCHOIDES FRAGARIAE: STUDIES ON FACULTATIVE FEEDING AND DESICCATION TOLERANCE

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THE FOLIAR NEMATODE *APHELENCHOIDES FRAGARIAE*:  
STUDIES ON FACULTATIVE FEEDING AND  
DESICCATION TOLERANCE

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A Dissertation  
Presented to  
the Graduate School of  
Clemson University

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In Partial Fulfillment  
of the Requirements for the Degree  
Doctor of Philosophy  
Plant and Environmental Sciences

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by  
Zhen Fu  
May 2012

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Accepted by:  
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## ABSTRACT

The foliar nematode *Aphelenchoides fragariae* is an economically important and frequent pest of ornamental crops. In nurseries and landscapes in the United States, foliar nematodes can be a serious problem affecting hostas (*Hosta* spp.), a common perennial ornamental plant that thrives in shady environments. Fundamental research on the biology and ecology of this nematode provides the basis for development of sound management strategies. The general objective of this dissertation was to study two behaviors that make foliar nematodes very successful as plant pests: desiccation tolerance and alternative feeding on fungi (or facultative feeding on the plant). To study the molecular basis of these behaviors, I used a transcriptomic approach complemented with *in vitro* and greenhouse experiments. As part of the greenhouse studies, I developed a standard protocol for the assessment of resistance to *Aphelenchoides fragariae* on hosta cultivars. I evaluated the effects of inoculum type (nematodes maintained on fungus vs. maintained on plants), inoculation method (with injury vs. without injury), and harvesting method to select the best procedures, and estimated the correlations between nematode reproduction and symptom severity. The variability of the correlation between symptoms and nematode reproduction highlighted the importance of measuring both parameters when evaluating cultivar resistance. More importantly, I demonstrated that there is a loss of virulence for inoculum maintained and increased on fungus cultures. For the molecular studies, I used Illumina technology and *de novo* assembly to complete a transcriptome of the nematode (a non-model organism lacking a reference genome) to

generate data corresponding to nematode mRNAs from mixed life stages under four different treatments (fungus diet, plant diet, diet changed from plant to fungus, and desiccation stress). This transcriptome is the first comprehensive sequence resource available for foliar nematodes. I present an annotated overview of the *Aphelenchoides fragariae* transcriptome and provide information on gene families, gene structure, potential gene functions, and gene pathways (molecular, cellular and biological). I identified and characterized a  $\beta$ -1,4-endoglucanase, which I named Afr-ENG-1, that is differentially expressed when the nematode feeds on fungi or plants. When individuals from hosta plants were transferred to a fungus culture, expression of the enzyme decreased 1,812- fold after five generations on the fungus diet. Diet was also associated with changes in nematode body size and in the severity of symptoms caused on hosta leaves. Plant-diet nematodes caused larger lesions and were longer and thinner than fungus diet nematodes. Full length sequences of *Afr-eng-1* were obtained and found to encode a glycosyl hydrolase family 5 (GHF5) protein. This is the first  $\beta$ -1,4-endoglucanase and plant-parasitism related gene described in the genus *Aphelenchoides*. For the desiccation tolerance experiments, *Aphelenchus avenae*, a model nematode for anhydrobiosis studies, was used as a reference. *Aphelenchoides fragariae* showed higher survival rates than *A. avenae* under desiccation and osmotic stress conditions. Two glutaredoxin (*glx*) and three trehalose phosphate synthase (*tps*) genes were identified in the *A. fragariae* transcriptome of nematodes subjected to desiccation conditions. Phylogenetic analysis showed that the Af-TPS was closely related to TPS of *A. avenae*. Expression of *Af-tps* and *Af-glx* suggests participation of anti-oxidation and cellular

membrane protection mechanisms in the desiccation tolerance mechanisms of foliar nematodes. These observations on the feeding behavior and desiccation tolerance of the foliar nematode improve our knowledge of the basic biology of this nematode and will enhance our ability to increase the arsenal of weapons to fight damage to plants from these parasitic nematodes.

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## INTRODUCTION

Foliar nematodes, *Aphelenchoides fragariae* (Ritzema Bos, 1890) Chrisite, 1932 (Aphelenchida: Aphelenchidae), sometimes known as Strawberry crimp nematodes, are endo- and ectoparasites that feed on aerial parts of plants. *Aphelenchoides fragariae* is a bisexual species and amphimixis (n=4) seems to be obligatory (Siddiqi, 1975). The life cycle is completed in 10-11 days at 18 °C in leaves of begonia (Strumpel, 1967). Over 250 plants in 47 botanical families are recorded as hosts (Siddiqi, 1975). Foliar nematodes are an economically damaging and frequent pest of nursery-grown crops including ferns, foliage and flowering plants, and herbaceous and woody perennials. Symptoms are vein-delimited lesions, chlorosis, necrosis, and defoliation, and can render plants unmarketable (Jagdale and Grewal, 2002; Kohl *et al.*, 2010; LaMondia, 1999).

In nurseries and landscapes in the United States, foliar nematodes can be a serious problem affecting hostas (*Hosta* spp.), a commonly grown herbaceous perennial ornamental that thrives in shady environments. The nematodes feed on mesophyll cells of hosta leaves, causing vein-delimited lesions that turn necrotic over time (Wallace, 1961). After penetrating, migrating, feeding, and propagating in the leaf tissue, foliar nematodes can overwinter in abscised leaves, dormant buds, or in the soil (Jagdale and Grewal, 2006). In the spring, nematodes migrate to the new leaves along the stem in thin films of water (Jagdale and Grewal, 2006). Nematodes can be disseminated to non-infected leaves by direct contact of foliage or by splashed water from irrigation or rain (Kohl *et al.*, 2010).



In order to develop adequate management strategies for this nematode on hostas, it is important to effectively combine the knowledge of cultivar susceptibility with the existing chemical and cultural control options (Jagdale and Grewal, 2002; Jagdale and Grewal, 2004; Jagdale and Grewal, 2006; LaMondia, 1999). However, there is currently no standardized method for assessing resistance of hosta cultivars to foliar nematodes. The objective of the first chapter of this dissertation is to develop a standard protocol for the assessment of resistance to *A. fragariae* on hosta cultivars. In order to select the best procedures, effects of inoculum type (maintained on fungus vs. maintained on plants) and inoculation method (with injury vs. without injury) on nematode reproduction and plant symptom were evaluated, and the efficiency of different harvesting methods was compared. More importantly, the correlations between nematode reproduction and symptom severity were estimated to determine if both parameters are necessary to assess the resistance of the hosta plants to foliar nematodes.

Limited nematicides are available for the control of foliar nematodes. Development of good management strategies of foliar nematode requires a sound knowledge of the basic biology of the organism. Additionally, foliar nematodes are interesting subjects of study because of their ability to survive low moisture conditions and the ability to feed on fungi as well as on plants. The mechanisms involved in both of these key adaptations have been poorly studied.

Nowadays sequencing transcriptomes and genomes using next-generation-sequencing (NGS) technology is becoming cost-efficient and labor-saving. In the past few years, NGS has largely improved our understanding of the complexity of gene expression and

splicing-regulation in both model and non-model organisms (Feldmeyer *et al.*, 2011; Wang *et al.*, 2010). Programs have been developed to assemble transcriptomes of non-model organisms without a reference genome (Grabher *et al.*, 2011). In the Aphelenchida family, only a few transcriptomic studies have been published: expressed sequence tags (EST) of *Aphelenchus avenae* (Karim *et al.*, 2009), EST of *Bursaphelenchus xylophilus* and *Bursaphelenchus mucronatus* (Kikuchi *et al.*, 2007). Sequence data with higher coverage and full-length of cDNA are necessary to elucidate the molecular mechanisms of important biological processes. In the second chapter of this dissertation, the objective is to use Illumina HiSeq 2000 to sequence the transcriptome of the foliar nematode *A. fragariae* under four different conditions (fungus diet, plant diet, diet-changed from plant to fungi, and desiccation stress). This will provide a tool that can be used to study the biology and physiology of the foliar nematode. Transcripts differentially abundant under the different conditions will be compared to find genes associated with facultative feeding and desiccation stress.

Facultative feeding might be related to the enzymes secreted by foliar nematodes. The esophageal gland cells of plant-parasitic nematodes (PPN) can secrete cellulases, which are injected via the stylet into the plant tissue (Davis *et al.*, 2004). Over the last decade, many PPN cell-wall-degrading enzymes have been identified and studied. Endo-1,4- $\beta$ glucanases (E.C 3.2.1.4), which degrade the  $\beta$ -1,4-linkage of cellulose, are some of the better characterized. Isolates of *B. xylophilus* that are more vigorous in the mycophagous phase have shown reduced virulence on pine (Wingfield, 1987). The mechanisms associated with feeding preferences have not been elucidated, and no

research has been published on the differential expression of nematode cellulases under different diets. The objectives of the third chapter are to identify and characterize a  $\beta$ -1,4-endoglucanase in *A. fragariae*, determine the expression levels of this gene under plant and fungus diets, and examine phenotypic differences associated with the different diets.

Foliar nematodes can overwinter in the soil, dormant crowns, and abscised leaves and then migrate to the new leaves in the spring (Jagdale and Grewal, 2006). In the winter, foliar nematodes may enter anhydrobiosis in the soil and resume activity when free moisture is available. This ability to survive desiccation might render this pathogen difficult to eliminate even after repeated nematicide applications, but it has not been studied in *A. fragariae*. Desiccation has been studied in detail in few nematode species: stem and bulb nematode *Ditylenchus dipsaci* and fungivore *A. avenae* (Burnell and Tunnacliffe, 2011). A number of genes have been detected associated with desiccation and osmotic tolerance in *A. avenae*, including glutaredoxin (Browne *et al.*, 2004) and trehalose phosphate synthase (Goyal *et al.*, 2005). The objectives of the fourth chapter are to test the survival ability of foliar nematodes under different stress conditions, and to examine the changes in gene expression associated with desiccation tolerance in *A. fragariae* using *A. avenae* as a comparative model. Transcriptome sequences of foliar nematode were be used to design gene specific primers and compare the results of the differential expression using quantitative reverse transcription PCR.

## CHAPTER ONE

### A PROTOCOL FOR ASSESSING RESISTANCE TO *APHELENCHOIDES*

#### *FRAGARIAE* IN HOSTA CULTIVARS

Foliar nematodes *Aphelenchoides fragariae* (Ritzema Bos, 1890) Christie, 1932 (Aphelenchida: Aphlenchidae) are endo- and ectoparasites of many plants, including ornamental and agricultural crops (De Waele, 2002; Siddiqi, 1975). In nurseries and landscapes in the United States, these nematodes can be a serious problem (Jagdale and Grewal, 2002; 2004; 2006) affecting hostas (*Hosta* spp.), a commonly grown herbaceous ornamental plant that thrives in shady environments. The nematodes can enter the foliar tissue through stomata or wounds (Jagdale and Grewal, 2006; Wallace, 1959) and feed on mesophyll cells (Sanwal, 1959; Wallace, 1959), causing characteristic vein-delimited lesions that start as lightly chlorotic and then turn necrotic. The nematodes may overwinter in the soil, dormant crowns and dry leaves (Jagdale and Grewal, 2006). In temperate regions, they migrate to the new leaves in the spring (Jagdale and Grewal, 2006).

Control of foliar nematodes on hosta can be difficult because of the survival behaviors of the nematode (De Waele, 2002; Jagdale and Grewal, 2006) and because hostas are popular perennial plants adapted to a wide geographical range, with numerous species and cultivars grown (Schmid, 1991). Because thousands of plants are traded each year, there is increasing concern among growers about the movement of this nematode and fear of dissemination to non-infested areas. Forty-seven countries have legislation regulating

the movement of this species in international trade (Lehman, 2004). In order to develop adequate management strategies, it is important to effectively combine the knowledge of cultivar susceptibility with the existing chemical and cultural control options (Jagdale and Grewal, 2002; 2004; 2006; La Mondia, 1999). However, there is currently no standardized method for assessing resistance of hosta cultivars to foliar nematodes. Jagdale and Grewal (2006) tested the pathogenicity of *A. fragariae* on 23 cultivars, and found it pathogenic on 20 of them. They measured presence or absence of symptoms, but did not assess symptom severity or nematode reproduction. Because the value of hostas lies mainly in the quality of their foliage, it is important to evaluate symptom severity. Symptom expression, however, can be highly variable due to influence of the environment and characteristics of the foliage of each cultivar. When symptom development is slow, plants supporting nematode reproduction may be asymptomatic (McCuiston ., 2007). Consequently, nematode reproduction becomes an important part of assessing plant resistance and should be measured along with symptom severity. Breeders and growers would benefit from having a standard protocol that assesses plant resistance, as defined both as reduced nematode reproduction (De Waele, 2002), and as reduced disease.

The objective of this study was to develop a standardized protocol for the assessment of resistance to *A. fragariae* on hosta cultivars. To select the best procedures, we evaluated the effects of inoculum type (maintained on cultured fungus vs. maintained on plants), inoculation method (with injury vs. without injury), and nematode harvesting

methods. We also explored potential correlations between nematode reproduction and symptom severity.

## **Materials and Methods**

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

***Nematode inoculum sources.*** The effect of two types of *A. fragariae* inoculum [one maintained on a fungus culture (‘fungus’) and one maintained on hosta plants (‘plant’)] on pathogenicity, symptom severity, and host suitability on the six hosta cultivars was evaluated. The ‘fungus nematodes’ are part of the Clemson University Nematode Collection and have been cultured *in vitro* on *Cylindrocladium* sp. grown on potato dextrose agar (PDA, HiMedia laboratories, India) under laboratory conditions for 25 years. The origin of the isolate is uncertain. The ‘plant nematodes’ were isolated from infected hostas in the South Carolina Botanical Garden, identified by morphology, and

cultured on hostas in the field and the greenhouse. To obtain the ‘fungus inoculum’, nematodes were extracted by Baermann funnel (Baermann, 1917) and a water suspension of mixed developmental stages from the extraction was adjusted to a concentration of 5,000 individuals per ml before inoculation. To obtain the ‘plant inoculum’, hosta leaves infected with *A. fragariae* were cut into 1-cm<sup>2</sup> pieces and soaked in water for 24 h. The mix was poured through nested sieves of 20 mesh (850 µm) and 500 mesh (25 µm) and the contents of the 500-mesh sieve were transferred to a Baermann funnel. The extract was washed several times with sterilized tap water and adjusted to the same concentration as the fungus inoculum. Each inoculum type was evaluated with each inoculation method (described below) on all six cultivars.

***Leaf inoculation methods.*** Two inoculation methods (with and without leaf injury) were evaluated. Each combination of inoculum type and inoculation method was replicated on five plants of each of the six cultivars. In all treatments, nematode inoculation was conducted on two arbitrarily selected leaves of each plant. One leaf was not injured prior to inoculation and the other was injured by one of two methods: with a scalpel, by making five short cuts in the upper side of the leaf, or with a needle, by making 10 perforations scattered between the leaf veins. Both leaves were wrapped with wet tissue paper (Kimwipes<sup>R</sup> 11cm x 21cm, Kimberly-Clark, Roswell, GA) and the suspension of the nematodes was carefully dispensed on the tissue paper. The plants were covered with black plastic bags after inoculation, in order to maintain a moist environment. The bags and tissue wrapping were removed after 72 h. All plants were kept in a shaded

greenhouse at  $25\text{ }^{\circ}\text{C} \pm 5\text{ }^{\circ}\text{C}$ . Treatments were arranged in a randomized complete block design and the experiment was performed twice.

***Data collection.*** Inoculated hosta leaves were harvested 35 days after inoculation. Symptom severity was evaluated by calculating the percentage of affected leaf area, using the grid method. Photographs of each inoculated leaf were taken and were later used to aid in the development of a rating key. The leaves were cut into  $1\text{-cm}^2$  pieces and soaked in tap water for 48 h at room temperature. The nematodes that emerged from the leaf pieces were recovered using nested sieves of 20 mesh (850  $\mu\text{m}$ ) and 500 mesh (25  $\mu\text{m}$ ) and counted.

***Comparison of efficiency of extraction methods.*** Three harvesting methods were evaluated: traditional Baermann funnel, modified Baermann funnel, and water-soaking. Symptomatic hosta leaves from different cultivars were collected, cut into  $1\text{-cm}^2$  pieces and mixed together. Leaf pieces were equally divided among treatments and weighed before processing. The traditional Baermann funnel method was evaluated at room temperature ( $22\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ ), while the modified Baermann funnel and the water-soaking methods were evaluated both at room temperature and at  $28\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ . Three replicates were included for each treatment. The amount of nematodes recovered after 24 h was counted and recorded. For the traditional Baermann funnel method, leaf pieces were wrapped with tissue paper (Kimwipes<sup>R</sup> 11cm x 21cm) and placed in a glass funnel filled with tap water. For the modified Baermann funnel method, leaf pieces were wrapped



with large Kimwipes<sup>R</sup> (37 cm x 42cm) and placed on a 20 mesh sieve (25 cm diameter, 850  $\mu\text{m}$  openings). The sieve was placed in a plastic container with tap water just covering the leaf material. A small aquarium pump was used to aerate the water during the incubation. For the water-soaking method, leaf pieces were placed in petri dishes (10 cm diameter) filled with tap water. The nematodes that emerged from the leaf pieces were recovered using nested sieves of 20 mesh (850  $\mu\text{m}$ ) and 500 mesh (25  $\mu\text{m}$ ) and counted.

***Statistical analysis.*** All nematode density data were natural log-transformed, except for those for the extraction method experiment. Nematode density, symptom severity, and extraction efficiency data were analyzed by One-way analysis of variance with JMP 9 software (SAS Institute, Cary, NC). Differences between treatments were determined by Fisher's Least Significant Difference or Student's *t*-test at  $P < 0.05$ . Potential correlations between symptom severity and nematode density were analyzed using the Bivariate fit procedure of JMP 9 software. If there were no differences between repeated experiments ( $P > 0.05$ ), data were combined for analysis.

## **Results**

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

***Effect of inoculum source.*** Both types of inoculum ('plant' and 'fungus') were pathogenic to all six cultivars tested, but there were differences ( $P < 0.05$ ) between the two types of inoculum in nematode reproduction and in disease severity (Fig. 1.1 and Table 1.1). Disease severity, measured as percentage of the leaf area with lesions, was higher in leaves treated with the 'plant' inoculum (Table 1.1). Characteristic, vein-delimited lesions were observed as early as 21 days after inoculation and progressed from yellow to brown in color as time passed. Overall, lesions were 2 to 8 times larger with 'plant' inoculum than with 'fungus' inoculum. The largest differences in disease severity as a result of inoculum type were observed with cultivar Patriot, where lesions were 10.34% of leaf area with 'plant' inoculum and 1.22% with 'fungus' inoculum. The disease severity data for cultivars Fragrant Bouquet and Golden Tiara were not included in Table 1.1 because the occurrence of other foliar disease symptoms compromised the reliability of the symptom severity evaluation. We did include the nematode density data for all six cultivars. On five of the six cultivars, nematode reproduction was higher in the leaves inoculated with 'plant' inoculum (Fig. 1.1). Only cultivar Albo Marginata supported higher nematode densities per leaf with the 'fungus' inoculum (Fig. 1.1). There were differences ( $P < 0.05$ ) between nematode densities obtained with 'plant' inoculum and with 'fungus' inoculum on Albo Marginata, Golden Tiara, Guacamole, and Patriot. For cultivars Fragrant Bouquet and Aureo Marginata, there were no statistical differences between the 'plant' and 'fungus' inocula. The highest nematode reproduction was observed on cultivar Aureo Marginata (17,400 individuals per leaf recovered 35 days after inoculation), using 'plant' inoculum.

***Effect of inoculation method.*** There were differences ( $P < 0.05$ ) in nematode reproduction and disease severity between inoculation treatments with and without injury (Fig. 1.2 and Table 1.2). Symptom severity was greater, when observed 35 days after inoculation, in leaves injured at the time of inoculation. The difference in disease severity was more noticeable with ‘plant’ inoculum than with ‘fungus’ inoculum. The combined mean size of the lesions was doubled for ‘fungus’ inoculum when aided by injury, but it is more than 8 times larger for ‘plant’ inoculum when aided by injury. Nematode reproduction was enhanced in a similar manner (Table 1.2). When combining the data for all cultivars, nematode densities were up to 38-fold higher on injured leaves, using ‘plant’ inoculum, and up to 3.7-fold higher on injured leaves, using ‘fungus’ inoculum.

***Comparison of efficiency of extraction methods.*** Numbers of nematodes extracted from the same weight of leaf material was different ( $P < 0.05$ ), depending on the extraction method used (Fig. 1.3). The traditional Baermann funnel technique yielded recovery of the lowest number of individuals, but it was the most consistent technique (lowest standard deviation) and the extracts also contained the least leaf debris. This is important because it makes the nematodes within the extracts easier to quantify. The most efficient extraction method tested was water-soaking, which yielded more than four times more nematodes than were obtained than with the traditional Baermann funnel technique. Incubation temperature did not change the efficiency of the water-soaking or the

modified Baermann funnel techniques. The modified Baermann funnel technique yielded extracts with the most debris and, consequently, were the most difficult to quantify.

***Correlation between disease severity and nematode density.*** For three of four cultivars, the severity of symptoms increased directly with nematode population, but the level of correlation varied by cultivar (Fig. 1.4). For cultivar Albo Marginata nematode population density and disease severity were not correlated. A low positive correlation ( $r = 0.538$ ;  $P < 0.05$ ) between these two variables was observed for Guacamole. Higher positive correlations were observed with cultivars Aureo Marginata ( $r = 0.734$ ;  $P < 0.05$ ) and Patriot ( $r = 0.856$ ;  $P < 0.05$ ). Equations were derived to describe relationships between population density of *A. fragariae* and symptom severity for the three cultivars with positive correlations (Fig. 1.4). In each, the slope of the line is a measure of the susceptibility of the cultivar. The most susceptible cultivar, i.e., the one with the largest lesions caused by a given number of nematodes, was Guacamole.

## **Discussion**

The use of resistant and tolerant cultivars is an important component of an integrated management plan for foliar nematodes on hosta. In order to identify tolerance and resistance in commercial hosta cultivars, reliable and efficient screening methods are required. Based on the results of the experiments of this study and on our experience working with this nematode, we recommend the following protocol for screening for resistance to foliar nematode on hosta cultivars:

#### A. Inoculum

1. Maintain and increase foliar nematode inoculum on hosta plants in the greenhouse.

We recommend Patriot and Guacamole for this purpose, because they are commonly grown cultivars that sustain adequate nematode reproduction.

2. For inoculum extraction, cut infected hosta leaves into 1-cm<sup>2</sup> pieces and soak in tap water for 48 h at room temperature. Pour the mix through nested sieves of 20 mesh (850 µm) and 500 mesh (25 µm). Wash contents collected on the 500 mesh sieve with tap water. Adjust the concentration of the extract to 5,000 mixed stage individuals per ml. and use within three days of extraction.

#### B. Inoculation procedure

1. Hosta plants to be evaluated should be potted individually, and each plant to be inoculated should have at least 8 leaves.
2. Select two healthy leaves on each hosta plant and make ten perforations, scattered between leaf veins, with a needle on both leaves. Wrap both leaves with wet tissue paper (e.g., Kimwipes<sup>R</sup> 11 cm x 21 cm). Dispense 1 ml of the suspension of nematodes on one leaf, and 1 ml of sterile tap water on the other leaf as negative control.
3. Cover the plants with black plastic bags. After 72 hours, remove the bags and tissue wrappings.

#### C. Experimental design

Use a completely randomized arrangement. Replicate each treatment at least 5 times.

Run the experiment twice. One susceptible cultivar should be included in each

experiment as positive control. Based on the results of this study, we recommend Guacamole as the susceptible control cultivar.

#### D. Environmental conditions

1. Maintain in the greenhouse at  $25\text{ }^{\circ}\text{C} \pm 5\text{ }^{\circ}\text{C}$ .
2. Use shade cloth (50% to 60% density) to provide shade for the plants.
3. Water the plants judiciously, avoiding splashing.
4. Provide light/dark phase of 12 hours, supplementing with artificial lights when necessary.

#### E. Data collection

1. Allow the test to run 35 days after the inoculation.
2. Collect inoculated leaves and assess symptom severity of each, using a 0-6 scale (Figure 1.5) based on the percentage of the total leaf area with lesions and chlorosis. Examples of rated leaves are provided in Figure 1.6.
3. Evaluate the positive and negative controls first. If the positive control is rated 0 or 1, discard the test and run again. If the negative control (i.e. the leaves inoculated with water) is not rated 0, discard the test and run again.
4. Cut inoculated leaves into  $1\text{ cm}^2$  pieces and soak in a 10 cm-diameter glass petri plate with 40 ml tap water for 48 h at room temperature. Retrieve the nematodes passing through nested sieves of 20 mesh (850  $\mu\text{m}$ ) and 500 mesh (25  $\mu\text{m}$ ) and count.
5. Report the numerical rating for symptom severity and the nematode density data for each plant and cultivar.

Differences in observed reproduction of nematode populations are often due to culture, preparation, and delivery of nematode inoculum to host materials (De Waele, 2002). There have been very few studies on the fate of inoculated nematodes, but loss – inoculated nematodes that do not invade the host – is believed to be very high. Plowright and Gill (1994) estimated that more than 75% of *Ditylenchus angustus* inoculum was lost. Our observations support that the use of the wet tissue and the plastic bag for inoculation helps minimize inoculum loss, although we have simply observed improved infection and have not quantified losses. These observations are also supported by the fact that a common cultural control recommendation for foliar nematodes is to avoid excess surface moisture of the foliage (Siddiqi, 1975).

It is convenient to rear nematodes in pure cultures either in fungus cultures or on callus tissue. Several migratory endoparasitic nematodes like *Pratylenchus* spp., *Radopholus* spp., and *Ditylenchus* spp. are routinely cultured on carrot disks or other *in vitro* methods. Some authors report changes in infectivity of the inoculum reared *in vitro* (Stoffelen *et al.*, 1999), while others report no differences (Elsen *et al.*, 2001; Hogger, 1969). Ali and Ishibashi (1996) recommend that infectivity and aggressiveness of *Ditylenchus* species reared in monoxenic culture be monitored on the field host. In contrast, Erikson (1972) reports having maintained and regularly subcultured on callus a lucerne (alfalfa) race of *Ditylenchus dipsaci* for more than 10 years, without losing host specificity and ability to cause symptoms.

In the case of *A. fragariae* for hosta infection, we have demonstrated a loss of virulence of inoculum maintained and increased on fungus cultures. Researchers who,

for convenience, decide to use inoculum reared on fungi should be aware that symptom expression and nematode reproduction can be greatly reduced. Additionally, culturing nematodes on fungi comes with the risk of inoculating fungal propagules along with the nematode inoculum (MacGuidwin and Slack, 1991). This is an important risk if we consider that fungi reported as used to culture *A. fragariae* can be pathogenic to plants: *Rhizoctonia solani* (Jagdale and Grewal, 2006), *Botrytis cinerea* (De Waele, 2002), and *Cylindrocladium* sp. (this study).

Wingfield (1987) observed that races of *Bursaphelenchus xylophilus* with low pathogenicity to pine could be more vigorous in the mycophagous phase. *Aphelenchoides fragariae* is closely related to *B. xylophilus* and similar to it in its ability to feed on both fungi and plants (De Waele, 2002; Jagdale and Grewal, 2006; Siddiqi, 1975), but distinct phytophagous and mycophagous phases of the foliar nematode life cycle have not been characterized. Fungal feeding in foliar nematodes is believed to play an important role in survival in the soil and plant debris, and it is possible that isolates with low pathogenicity are more vigorous when feeding on fungi than when feeding on plants. This hypothesis is yet to be tested.

We recommend injuring the hosta leaves with a needle as part of the inoculation procedure. Our research showed that foliar nematodes will infect hosta plants without the aid of injury, but that certain cultivars will show few to no symptoms without injury. Jagdale and Grewal (2006) showed that injury was necessary to cause symptoms on cultivars like ‘Fried Green Tomatoes’ and ‘Fragrant’. In our study, we found the same was true for Patriot. We recommend small perforations (>1mm) made with a sharp



needle. In the control mock-inoculations (with water only), these perforations healed by the time of data collection, after 35 days. For cultivars with more succulent leaves, injury with scalpel (cuts) should be avoided, as we observed that these are readily colonized by opportunistic fungi. Because injury cannot be completely avoided in commercial settings (transporting, cultural practices, etc.) or in nature (wind, insects, etc.), we believe that it should be part of the standard protocol.

We recommend the water-soaking method at room temperature for extraction of foliar nematodes from leaf tissue. This method is the simplest and yielded adequate amounts of nematodes free from leaf tissue debris. The traditional Baermann funnel technique yielded a cleaner extract and also required little labor and simple equipment, but the extraction efficiency was lower. Fortuner (1991) reported that, with this extraction method, specimens can be trapped by the tissue and the sides of the funnel. We were expecting the extraction efficiency to be improved by aeration (modified Baermann), as oxygenation of the water has been reported to improve migration of nematodes from the plant tissue (Griesbach *et al.*, 1999; Hirling, 1971), but we did not observe such improvement. It is possible that aerating favored the growth of fungi and bacteria that could affect nematode motility.

Reports of differences in host response and nematode reproduction between series of experiments conducted under similar conditions are not uncommon in nematology (De Waele and Elsen, 2002). Such differences may arise from differences in the environmental conditions, developmental stage of plants, and infectivity of the inoculum. The occurrence of these differences underlines the importance of including the same

susceptible control host in each experiment. This way, comparison of results obtained from different tests is possible.

The variability of correlation between symptom expression and nematode reproduction that our results showed, indicates the importance of measuring both parameters when evaluating cultivars. Even when we found correlations, the  $R^2$  values of these equations, were not high enough to adequately predict lesion sizes with population densities or vice versa. Our suggested protocol assesses both resistance (reduced nematode reproduction) and tolerance (reduced loss of aesthetic value caused by symptom expression, regardless of nematode reproduction), and provides a useful tool for breeders, nematologists, consultants, and extension specialists in the development of new cultivars and of recommendations regarding cultivar selection.

Table 1.1. Severity of symptoms, measured as percentage of leaf area with lesions, caused by *Aphelenchoides fragariae* maintained on hosta plants (plant inoculum) or. on *Cylindrocladium* sp. (fungus inoculum), on four hosta cultivars ( $n=10$ ).

| Type of inoculum | Symptom severity by cultivar <sup>a</sup> |                 |                 |                 |
|------------------|---|-----------------|-----------------|-----------------|
|                  | Albo Marginata                            | Aureo Marginata | Guacamole       | Patriot         |
| Plant inoculum   | 10.34% ±1.79% A                           | 4.17% ±1.51% A  | 25.95% ±0.85% A | 10.34% ±3.43% A |
| Fungus inoculum  | 4.13% ±0.75% B                            | 1.21% ±0.67% B  | 3.53% ±0.71% B  | 1.22% ±0.55% B  |

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

Table 1.2. Severity of symptoms, measured as percentage of leaf area with lesions, caused by *Aphelenchoides fragariae* maintained on hostas (plant inoculum) vs. maintained on *Cylindrocladium* sp. (fungus inoculum), and inoculated on leaves with and without mechanical injury. Data for six cultivars were combined ( $n=30$ ).

| <b>Symptom severity by inoculum source (% of leaf area with lesions)<sup>a</sup></b> |                       |                        |
|--|-----------------------|------------------------|
| <b>Inoculation method</b>  | <b>Plant inoculum</b> | <b>Fungus inoculum</b> |
| Injured <sup>b</sup>   | 22.53% ± 5.76% A      | 3.23% ± 0.52% A        |
| Non-injured  | 2.68% ± 0.76% B       | 1.60% ± 0.47 % B       |

<sup>a</sup> Different letters indicate significant differences ( $P < 0.05$ ) within the same column (inoculum type) according to Student's  $t$ -tests.

<sup>b</sup> Injuries were made with a needle (10 perforations per leaf).

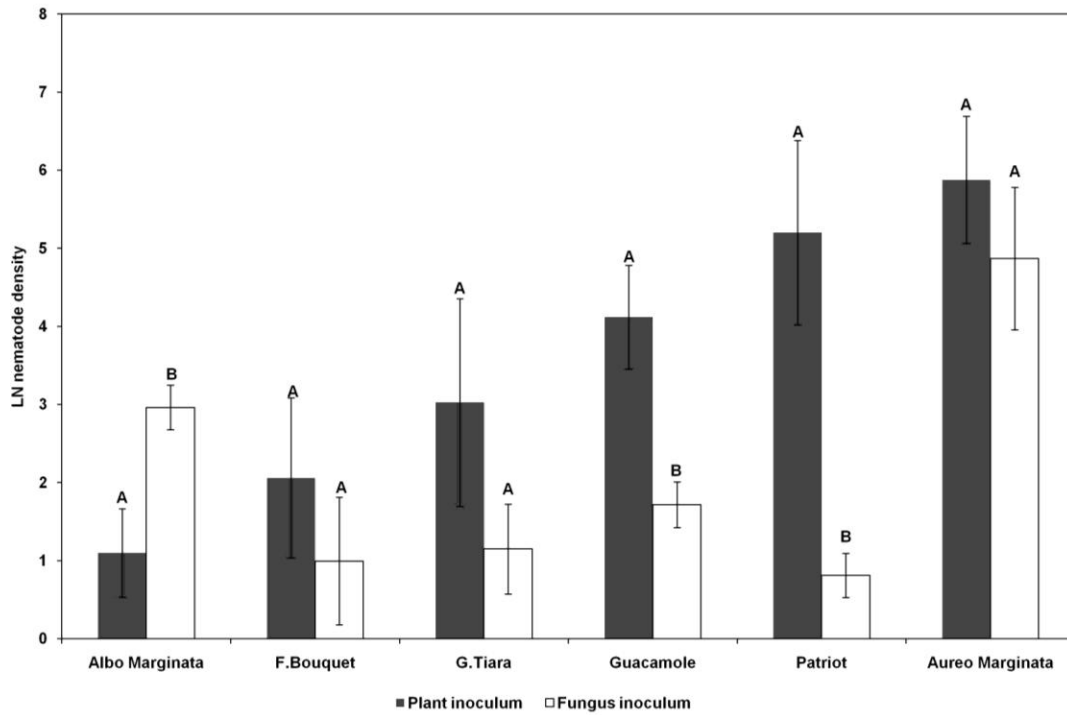


Figure 1.1. Effect of *Aphelenchoides fragariae* inoculum type (maintained on fungus or maintained on plants) on nematode density on six hosta cultivars: Albo Marginata, Fragrant Bouquet, Golden Tiara, Guacamole, Patriot and Aureo Marginata. Error bars are standard error of the mean ( $n=20$ ). Different letters indicate significant ( $P < 0.05$ ) differences within the same cultivars according to Student's  $t$ -test.

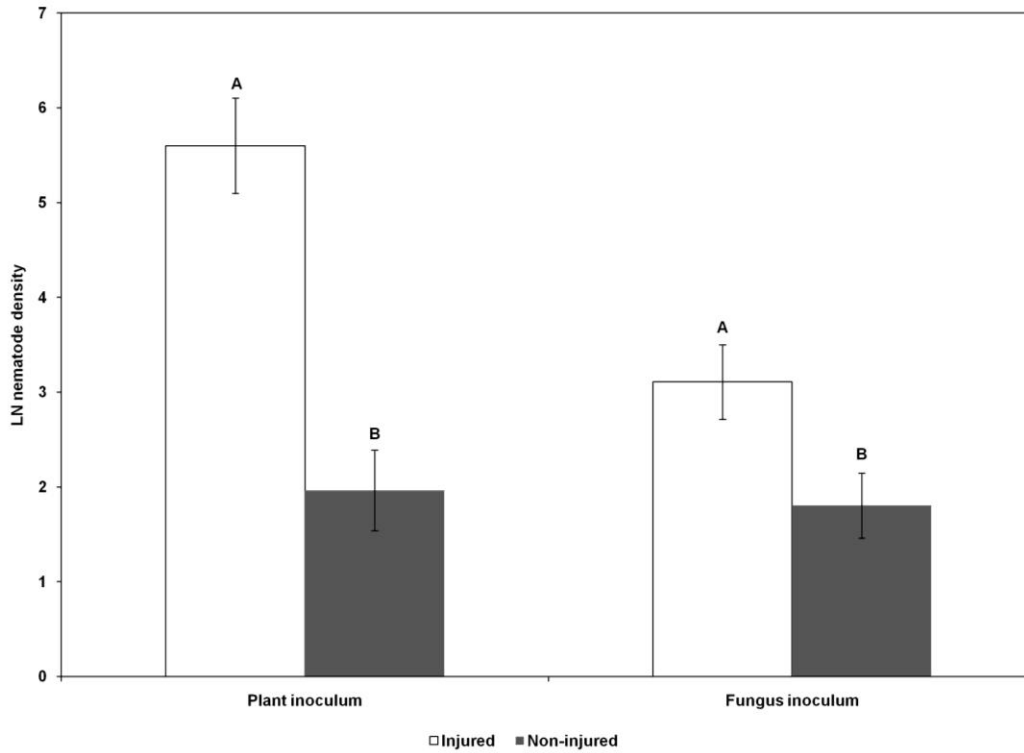


Figure 1.2. Effect of injury on *Aphelenchoides fragariae* density following application of two types of inoculum (maintained on fungus or maintained on plants). Error bars are standard error of the mean ( $n=44$ ). Different letters indicate significant ( $P < 0.05$ ) differences within the same inoculum type according to Student's  $t$ -test.

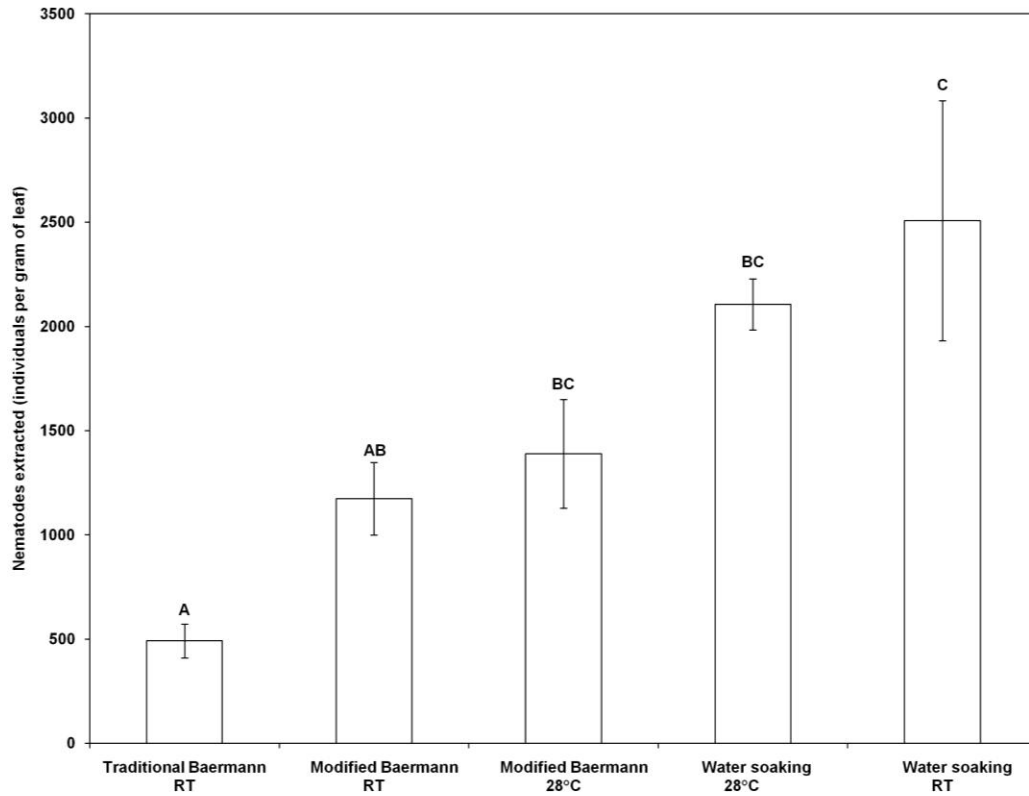


Figure 1.3. Comparison of five extraction methods for recovery of *Aphelenchoides fragariae* individuals from hosta (*Hosta* spp.) leaves. Error bars are standard errors of the means. Extraction methods not sharing a common letter indicate significant ( $P < 0.05$ ) differences according to Fisher's Least Significant Difference ( $P < 0.05$ ).

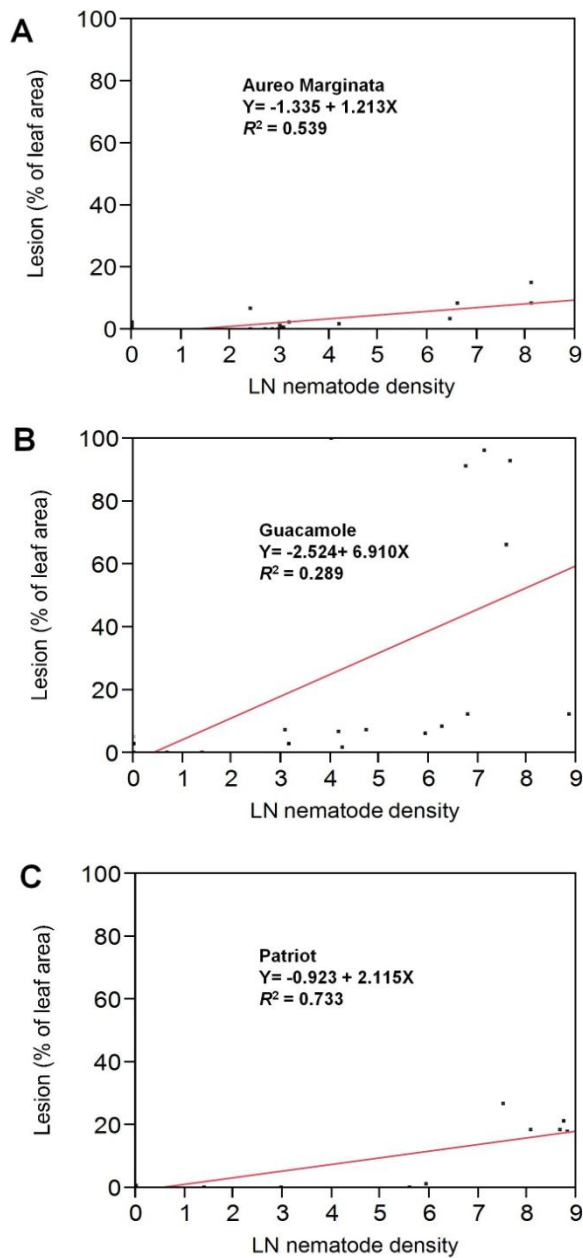


Figure 1.4. Regression analysis of symptom severity (expressed as percentage of leaf area with lesions) caused by *Aphelenchoides fragariae* vs. nematode density on three hosta cultivars: A. Aureo Marginata; B. Guacamole; C. Patriot



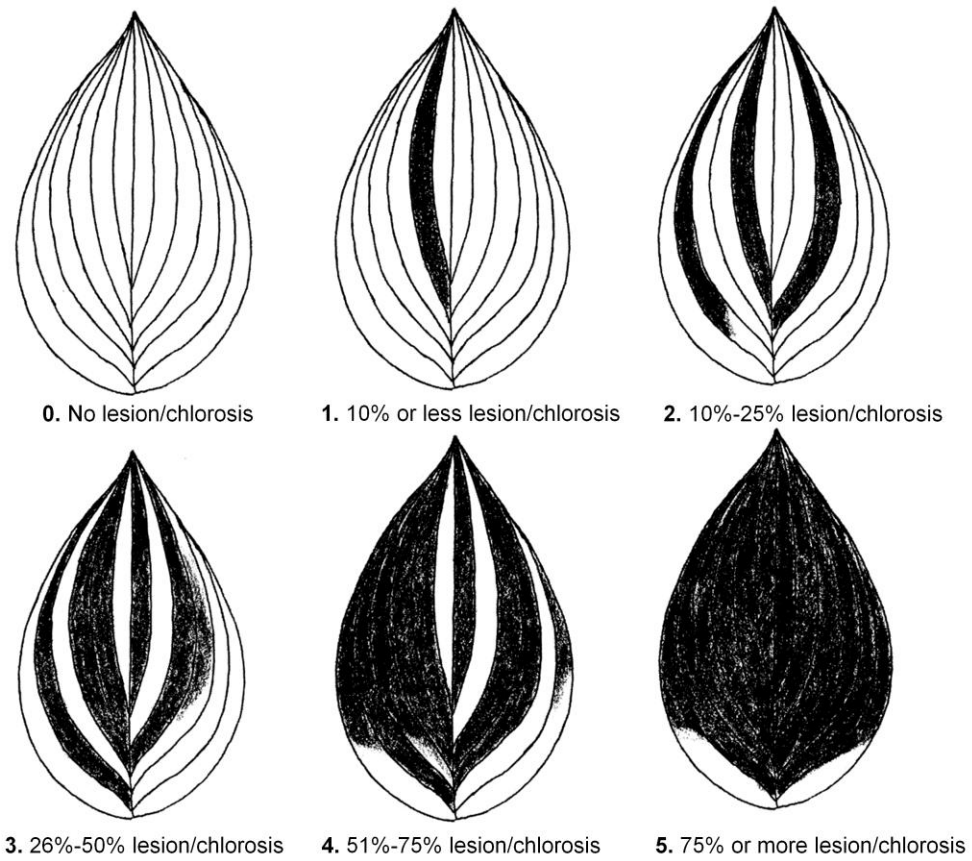


Figure 1.5. Rating chart of symptom severity on hosta caused by foliar nematode (*Aphelenchoides fragariae*). Drawings of hosta leaves are based on appearance of cultivar Guacamole.

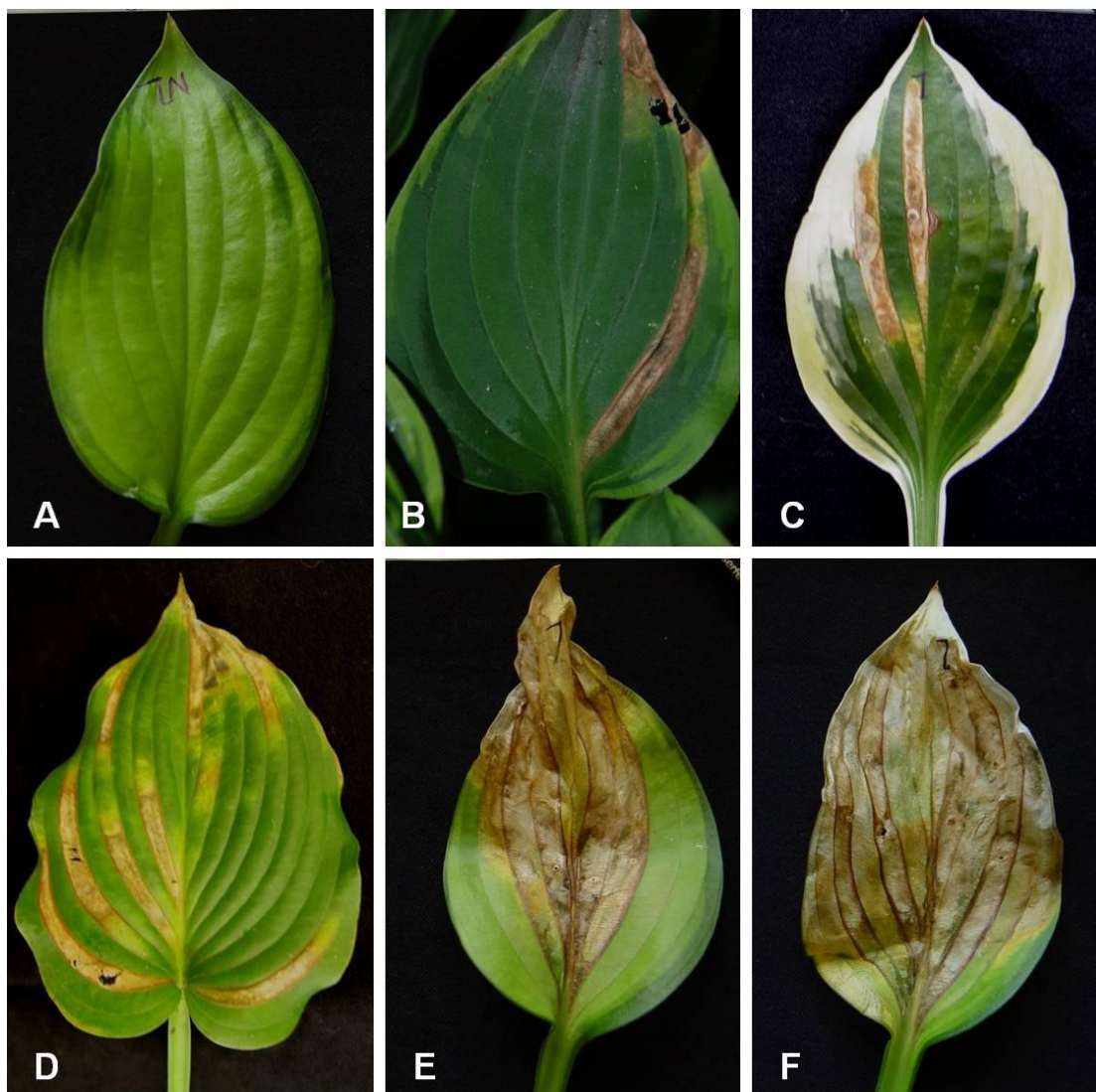


Figure 1.6. *Hosta* (*Hosta* spp.) leaves illustrating the different degrees of foliar nematode (*Aphelenchoides fragariae*) symptom severity, according to our suggested rating chart.

A. Leaf of cv. Guacamole, rated '0'. B. Leaf of cv. Golden Tiara, rated '1'. C. Leaf of cv. Patriot, rated '2'. D. Leaf of cv. Green Piecrust, rated '3'. E. Leaf of cv. Guacamole, rated '4'. F. Leaf of cv. Guacamole, rated '5'.

## CHAPTER TWO

### *DE NOVO* ASSEMBLY OF THE TRANSCRIPTOME OF THE PLANT-PARASITIC NEMATODE *APHELENCHOIDES FRAGARIAE*

Plant-parasitic nematodes (PPNs) are one of the major types of pests that threaten agricultural systems. Development of new management strategies is imperative with the banning and limitations of chemical nematicide and soil fumigant usage. The discovery and implementation of alternative management strategies require a sound knowledge of the basic biology of PPNs. The foliar nematode *Aphelenchoides fragariae* is an important pest of a variety of crops (Kohl *et al.*, 2010), but limited nematicides are available for its control. Additionally, foliar nematodes are interesting subjects of study because of their ability to survive low moisture conditions and the ability to feed on fungi as well as on plants. The mechanisms involved in both of these key adaptations have been poorly studied.

A large number of transcriptomes and genomes have become available during the last decade. In the nematode phylum, model organism *Caenorhabditis elegans* was the first organism to be sequenced completely (Wilson *et al.*, 1994), which greatly improved the understanding of the complexity of growth and development of nematodes. Sequencing the genome of root-knot nematode *Meloidogyne incognita* (Abad *et al.*, 2008) revealed a large number of plant-parasitism associated genes, which provide valuable information for future development of plant resistance against nematodes. Very few transcriptomic studies have been published for nematodes in the family Aphelenchoididae. Karim and

collaborators (2009) studied genes encoding cell-wall-degrading enzymes from *Aphelenchus avenae* using expressed sequence tag (EST), and the same technique has been used to sequence the pine wood nematode *Bursaphelenchus xylophilus* and *B. mucronatus* (Kikuchi *et al.*, 2007). In both studies on *Bursaphelenchus*, less than 5,000 contigs were obtained, and a portion of the contigs were not full-length sequences, which did not represent the whole transcriptome and splicing isoforms of genes. Sequence data with higher coverage and full-length of cDNA is necessary to elucidate the molecular mechanisms of important biological processes, such as facultative feeding and desiccation tolerance.

Next-Generation-Sequencing (NGS), which includes Roche/454, ABI SOLiD and Illumina, is a cost-efficient and labor-saving technique for advanced research in many fields, especially for *de novo* assembly of transcriptomes of non-model organisms without a reference genome (Feldmeyer *et al.*, 2011; Wang *et al.*, 2010). In the past few years, NGS has largely improved our understanding of the complexity of gene expression and splicing-regulation in both model and non-model organisms. A large number of transcriptome sequencing of non-model organisms has been done based on the Illumina platform due to its deeper coverage even though its read-length is shorter than that of 454 sequencing. However, the latest Illumina HiSeq 2000 can obtain up to 100 bp length read.

In this study, the objective was to use Illumina HiSeq 2000 to sequence the transcriptome of the foliar nematode *A. fragariae* under different conditions, to provide a tool that can be used to study the biology and physiology of this nematode.

## **Materials and Methods**

***Nematode materials:*** *Aphelenchoides fragariae* from four different conditions (fungus diet, plant diet, diet-changed, and desiccated) were included in this study. All conditions included a mixture of individuals of all developmental stages. The nematodes from the fungus diet condition were obtained from the Clemson University Nematode Collection and have been cultured *in vitro* on *Cylindrocladium* sp. grown in potato dextrose agar (PDA, HiMedia laboratories, India) under laboratory conditions for 25 years. The origin of the isolate is uncertain. The nematodes were extracted from the fungus cultures using a Baermann funnel (Baermann, 1917). The nematodes from the plant diet condition were cultured on hosta plants in the greenhouse and were harvested by soaking small pieces of leaves in water for 24 h. and recovered by pouring the extract through nested sieves of 20 mesh (850  $\mu\text{m}$ ) and 500 mesh (25  $\mu\text{m}$ ). The extract was transferred to a Baermann funnel and washed three times with sterilized tap water. The nematodes from the diet-changed condition were obtained by transferring approximately 1,000 individuals from the plant diet to a fungus culture. For this, the nematodes were washed three times with 0.5% streptomycin sulfate (MP Biomedicals, OH, USA), washed once with 0.5% chlorhexidine diacetate hydrate (Acros Organic, NJ, USA), and rinsed in sterilized tap water twice. Surface-sterilized nematodes were transferred to the *Cylindrocladium* sp. culture on PDA. The cultures were kept in an incubator at  $25\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ . The individuals for the desiccated condition were maintained on the fungus *Cylindrocladium* sp., harvested using Baermann funnel, washed three times with sterilized tap water and were

kept in sterilized tap water till RNA extraction. Approximately 50,000 nematodes in 20 ml sterilized tap water were vacuum filtered onto a 4.7 cm diameter Nuclepore membrane (5  $\mu\text{m}$  pores, Whatman, NJ) placed on a holder, and the membrane and holder were placed in the middle of a Nalgene filter funnel with clamp (Nalgene, NY), and a vacuum flask connected to the funnel was attached to an air pump. After the vacuum, only nematodes were left on the membrane. The membrane with the nematodes was transferred to an uncovered petri dish placed on a ceramic holder in an air-tight glass chamber with 72% glycerol solution in the bottom. Foliar nematodes formed pellets after 24 h incubation and were collected into a microcentrifuge tube, ready for use for total RNA extraction.

***RNA extraction.*** Approximately 5,000 nematodes of each of the conditions described above were used for RNA extraction, using the PureLink RNA mini Kit (Ambion, CA) and following the manufacturer's instructions. Total RNAs were treated with RNase-Free DNase set (Qiagen, Maryland, USA) to remove DNA contamination. Total RNA purity and degradation were checked on a 2% agarose gel before proceeding. RNA quality and integrity were verified using a 2100 Bioanalyzer with RNA nano chip (Aligent, CA). The samples from all the conditions had RNA Integrity Number (RIN) above 6.0 and concentration above 150 ng/ $\mu\text{l}$ .

***cDNA library construction and sequencing.*** Illumina sequencing using HiSeq 2000 was performed by the David H. Murdock Research Institute (Kannapolis, NC,

<http://www.dhmri.org/home.html>). The samples of the four conditions for sequencing were prepared using TruSeq RNA sample preparation kit (Illumina, San Diego, USA). Briefly, the poly-T oligo-attached magnetic beads were used to purify the mRNA from the total RNA. After purification, mRNAs were fragmented into small pieces using divalent cations. The small pieces of mRNA were synthesized into first strand cDNA using reverse transcriptase and random primers. The second strand cDNA synthesis was conducted using DNA Polymerase I and RNase H. Following double strand cDNA synthesis, the cDNA went through end-repair process, single 'A' base was added to the 3' ends, then the adaptors were ligated to the cDNA fragments. The cDNA fragments were purified and enriched with PCR to generate libraries and ready for sequencing. Libraries of the four conditions were 'tagged' using different oligonucleotides. The cDNA libraries were sequenced using Illumina HiSeq 2000 in one lane. The read length was 100 bp, and program was set to single-end sequence. Quality values of the sequences were calculated using Illumina Pipeline 1.5.

***Data filtering and de novo assembly.*** Sequences of each condition were cleaned by removing over represented sequences predicted by Fastqc (<http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc>), using TagCleaner (Schmieder *et al.*, 2010). Low quality sequences were removed with TagCleaner (Schmieder *et al.*, 2010). Quality of cleaned reads was checked using PRINSEQ (Schmieder and Edwards, 2011) to make sure there were no over-represented sequences. All reads from the four conditions were combined and assembled in the downstream de novo assembly pipeline.

*De novo* assembly was carried out using Trinity (Grabher *et al.*, 2011) on a node of the Palmetto Cluster (<http://citi.clemson.edu/hpc>) with four core processors and 512 GB memory. The assembled contigs were condensed by removing the alternative splicing isoforms, and in this chapter they are referred to as unigenes.

**Gene annotation.** All the unigenes assembled using Trinity (Grabher *et al.*, 2011) were exported and queried using Blast2GO (Conesa *et al.*, 2005) against Non-redundant (nr) database using the tblastx algorithm with a cutoff E value  $< 10^{-6}$ . All the reads with BLAST hits were annotated using Blast2GO with E value  $< 10^{-6}$ . All the unigenes were run through InterProScan using Blast2GO (Conesa *et al.*, 2005). The statistics of BLAST results, annotation results, and InterProScan were analyzed using Blast2GO (Conesa *et al.*, 2005).

**Differential expression analysis.** Trinity-generated contigs (including splicing isoforms) were used as references in the RSEM (<http://deweylab.biostat.wisc.edu/rsem/>). The reads of the plant condition were compared to the diet-changed condition, and the desiccated condition was compared to the fungal condition, which was in a hydrated state before RNA extraction. The expression value of each comparison was calculated using RSEM and DESeq (Anders and Huber, 2010).



## Results

**Raw data and de novo assembly.** Illumina HiSeq2000 generated 43,364,338 sequences from all four conditions in one lane. The mean length of the reads was 100 bp (Table 2.1). The average GC content was 42%. More reads were generated by Illumina on the desiccated condition than for the other three conditions (Table 2.1). Condensing of the assembled contigs removed 45,244 contigs, which were alternatively spliced isoforms. The mean length of the unigenes was 605 bp and ranged from 201 bp to 10870 bp. The average GC content of unigenes was 39.4% (Table 2.1).

**BLAST results.** Most of the BLAST hits had an E value ranging from  $E^{-6}$  to  $E^{-50}$  (Fig. 2.1). There were approximately 15,000 hits with extremely low E value ( $E < e^{-150}$ , Fig. 2.2). The top 5 species of all BLAST hits were all in the nematode phylum (*Caenorhabditis elegans*, *C. briggsae*, *C. brenneri*, *C. remanei* and *Ascaris suum*, Fig. 2.2). For the top hits of the each unigene, 8,239 genes were from *A. suum* and 2,665 genes from *Loa loa*, both animal-parasitic nematodes. *Bursaphelenchus xylophilus* (132 hits) and *Heterodera glycines* (68 hits) were also in the list of the Top-hit species distribution (Fig. 2.3).

**Annotation.** Of all 50,686 unigenes assembled by Trinity, 20,558 were annotated using Blast2GO (Figure 2.4), and 24,297 unigenes did not have BLAST hits. Of the sequences that had BLAST hits, 2,220 unigenes did not have mapping results, and 3,598 were not annotated. In total, 109,158 GO terms were retrieved, 71,983 of biological process,

26,467 of cellular component, and 10,760 of molecular function (Fig. 2.5). Primary metabolic processes created the largest percentage of the biological GO terms (Fig. 2.6). Most of the GO terms of cellular components were in the intracellular and intracellular part subcategory (Fig. 2.7) among the GO terms assigned to molecular function, DNA binding, transferase activity and transferring phosphorus containing groups, and receptor activities were the top three subcategories (Fig. 2.8).

**Differential gene expression.** Some genes associated with redox function of the cells were abundantly represented in the foliar nematode RNA-seq under desiccation compared to the hydrated control (Table 2.2), like butanediol dehydrogenase, aldo-keto reductase, and glutaredoxin. Meanwhile, Chaperonin containing tcp 1 delta subunit was in the list. Expression of some carbohydrate metabolite genes were highly reduced after 5 generations on a fungus diet (Table 2.3), like beta-endoglucanase and glucosamine-fructose-6-phosphate aminotransferase.

## Discussion

The most closely related species that been sequenced (EST) were *A. avenae* (Karim *et al.*, 2009) and *B. xylophilus* (Kikuchi *et al.*, 2007). Both of them share similar facultative feeding strategies (feeding on plants and fungi) with *A. fragariae*, and the latter is morphologically very similar to *A. fragariae*. RNAs from four conditions were sequenced independently in one lane of Illumina HiSeq 2000, however sequence yields on the Illumina platform, were not equal for all four conditions. This could be attributed

to differing amounts of cDNA library input in the flow cell. However, the high coverage of the sequencing was good enough to detect the low copy transcripts. Thirty million 35-bp length reads were sufficient to cover 90% of the genes from yeast cells *Saccharomyces cerevisiae* (Nagalakshimi *et al.*, 2010). In our case, more than 40 million 100 bp reads were obtained from the Illumina Hiseq, which we believe is sufficient to cover a large percentage of the whole transcriptome.

The advantage of Trinity is that it provides the information of the alternatively spliced isoforms, which is a big challenge in *de novo* transcriptome assembly, since multiple transcripts of one locus are common in Eukaryotes (Grabher *et al.*, 2011). Of 50,686 unigenes extracted from the assembled contigs using Trinity, only 52% of the contigs showed homology to known genes or protein in the non-redundant database. The other 48% of the contigs might be artifacts of short read and non-paired read assembly (Paszkiwicz and Studholme, 2010), or novel sequences.

Comparing to other plant-parasitic nematode expressed sequence tags (EST) projects: 43% of the *Ditylenchus africanus* EST did not have homology (Haegeman *et al.*, 2009), and 27% for *B. xylophilus* (Kikuchi *et al.*, 2007). The number of unigenes without homology was high in our study. The obvious reason is more unigenes were obtained from RNA-seq via Illumina instead of EST using traditional Sanger sequencing, with which only 4,847 ESTs were generated by Haegeman and his colleagues' (2009) study with *D. africanus*. Among the BLAST hits of unigenes, most of them were from nematodes, including the model nematodes: *C. elegans*, *C. briggase*, *C. brenneri*, and mammal parasite *A. suum* and human parasite *Brugia malayi*. These nematodes have

been the subjects of intense study. The reason that *Meloidogyne* spp sequences are not on the lists of Fig. 2.2 and Fig. 2.3 is that transcriptome or genome projects of *Meloidogyne* spp. have not been deposited into the non-redundant database.

In this study, 20,558 unigenes were annotated, and an average of 5.6 Gene Ontology (GO) terms were assigned to each annotated unigene (Fig. 2.5). Biological process was the biggest category of the GO terms assigned to the unigenes, the next category was molecular function and then cellular component.

Genes associated with redox system in the cells (e.g. Glutaredoxin) were up-regulated in the desiccation condition. It has been shown that glutaredoxin showed negligible re-regulation in the desiccation and osmotic stress in the anhydrobiotic nematode *Aphelenchus avenae* (Browne *et al.*, 2004). A putative chaperonin protein was also found up-regulated by desiccation, which suggests that the anti-aggregation role of chaperons could be involved in tolerance to desiccation stress (Chakrabortee *et al.*, 2010). Genes related to carbohydrate metabolism were down regulated after maintaining nematodes for 5 generations on a fungus diet, suggesting different metabolic pathways may be operating with different dietary demands. These putative genes might be good candidates for the study of pathogenicity factors in plant parasitic nematode infections.

Trans-splicing is a common RNA splicing mechanism for many primitive animal species, including nematodes (Bonen, 1993). It has been found that 70% of the *C. elegans* gene transcripts are trans-spliced with a splice-leader 1 or splice-leader 2 (Allen *et al.*, 2011). The desiccation model nematode, *Aphelenchus avenae*, which belongs to the same family as the foliar nematode, was also found to exhibit trans-splicing of its gene

transcripts (Goyal *et al.*, 2005), and four types of variation of splice-leader 1 (SL1) sequences were observed (Goyal *et al.*, 2005). We searched our contigs with these splice leader sequences, and we noticed that a large number of the transcripts have SL1 sequence at the 5' end. We plan to reassemble the transcriptome with reads cleaned of the SL1 and SL2 sequences, and we are interested to test the transcriptome qualities between assemblies from the reads with SL sequences trimmed and assembly from the raw reads. We will also investigate assemblies with different stringency and length of the trimming parameters. Thus, this chapter presents comprehensive information about the foliar nematode transcripts and provides a novel methodology to process Illumina RNA-seq data of the nematodes.

The transcriptome is a valuable resource to expand our inventory of knowledge of gene expression, RNA splicing, and metabolic pathways. We provide the first comprehensive sequence resource available for foliar nematodes. I present an annotated overview of the *Aphelenchoides fragariae* transcriptome and trust that this effort will facilitate future research of this nematode.

Table 2.1. Summary of raw reads and assembled contigs for the transcriptome sequences of *Aphelenchoides fragariae* representing mixed life stages subjected to four conditions (plant diet, fungus diet, diet-changed, and desiccated).

|                          | Number of<br>reads/contigs | Mean length<br>(bp) | N50 <sup>a</sup><br>(bp) | GC content<br>(%) |
|--------------------------|----------------------------|---------------------|--------------------------|-------------------|
| <b>Raw reads</b>         |                            |                     |                          |                   |
| Desiccated               | 15,963,132                 | 100                 |                          | 42                |
| Plant diet               | 8,250,953                  | 100                 |                          | 42                |
| Fungus diet              | 8,137,400                  | 100                 |                          | 42                |
| Diet-changed             | 11,012,853                 | 100                 |                          | 42                |
| <b>Assembled contigs</b> |                            |                     |                          |                   |
| Before condensing        | 95,930                     | 522                 | 658                      | 38.9              |
| After condensing         | 50,686                     | 605                 | 800                      | 39.4              |

<sup>a</sup> N50 contig size is a weighted median value and defined as the length of the smallest contig S in the sorted list of all contigs where the cumulative length from the largest contig to contig S is at least 50% of the total length.

Table 2.2. List of abundantly represented transcripts in *Aphelenchoides fragariae* RNA-seq under desiccation condition.

| Contig No.   | Tentative annotation <sup>a</sup>                            | Fold change <sup>b</sup>    |
|--------------|--|-----------------------------|
| Contigp23813 | WD40 repeat protein  | Not detected in the control |
| Contigp24369 | Peptidase M8, leishmanolysin                                 | Not detected in the control |
| Contigp16476 | Pyridoxamine 5 -phosphate oxidase                            | Not detected in the control |
| Contigp11974 | Lysyl-tRNA synthetase, class II                              | Not detected in the control |
| Contigp19800 | Calcium ion binding  | Not detected in the control |
| Contigp7407  | xyppx repeat family protein                                  | Not detected in the control |
| Contigp14796 | C2 calcium-dependent membrane targeting                      | Not detected in the control |
| Contigp502   | Butanediol dehydrogenase                                     | 168 up-regulated            |
| Contigp1209  | Aldo-keto reductase  | 161 up-regulated            |
| Contigp713   | Fad dependent oxidoreductase                                 | 84 up-regulated             |
| Contigp20644 | Cystathionine gamma-lyase                                    | 78 up-regulated             |
| Contigp143   | Adenosylhomocysteinase                                       | 75 up-regulated             |
| Contigp16523 | Seryl-tRNA synthetase, class IIa                             | 74 up-regulated             |
| Contigp17153 | Omega-6 fatty acid desaturase                                | 72 up-regulated             |
| Contigp6270  | Cullin 3   | 71 up-regulated             |
| Contigp19024 | Chaperonin containing tcp1 delta subunit                     | 55 up-regulated             |
| Contigp4407  | Cytochrome c oxidase assembly protein                        | 53 up-regulated             |
| Contigp1745  | Pyrroline-5-carboxylate reductase                            | 27 up-regulated             |
| Contigp11546 | Trehalase family protein                                     | 17 up-regulated             |
| Contigp10292 | <i>Caenorhabditis remanei</i> cytochrome P450 family protein | 8.5 up-regulated            |
| Contig778    | Glutaredoxin   | 3.2 up-regulated            |
| Contig8670   | Trehalose phosphate synthase                                 | 3.2 up-regulated            |

<sup>a</sup> Tentative annotation is based on the most significant BLAST alignment.

<sup>b</sup> Fold change for each contig is calculated by dividing the abundance of reads for the contig in desiccated condition by the reads for fungus diet (non-desiccated control) condition.

Table 2.3. List of down-regulated transcripts expressed in *Aphelenchoides fragariae* RNA-seq under diet-change condition compared to plant diet condition.

| Contig No.  | Tentative annotation <sup>a</sup>                       | Fold change <sup>b</sup>     |
|-------------|---|------------------------------|
| Contig31038 | <i>Caenorhabditis remanei</i> -IFB-1 protein            | Not detected in diet-changed |
| Contig40240 | Novel calcium channel family member                     | Not detected in diet-changed |
| Contig40380 | Insulin-like growth factor-binding protein              | Not detected in diet-changed |
| Contig40627 | Glucose transporter member                              | Not detected in diet-changed |
| Contig1197  | <i>Caenorhabditis remanei</i> -VIT-2 protein            | 176514 down-regulated        |
| Contig1935  | RAC gtpase-activating protein 1                         | 144352 down-regulated        |
| Contig1902  | Beta-endoglucanase                                      | 11291 down-regulated         |
| Contig11521 | High mobility group protein                             | 5122 down-regulated          |
| Contig17107 | Innexin unc-7   | 3667 down-regulated          |
| Contig8445  | Cathepsin z   | 1017 down-regulated          |
| Contig5945  | Fructose-bisphosphate aldolase a                        | 301 down-regulated           |
| Contig12877 | Isochorismatase family protein                          | 135 down-regulated           |
| Contig19317 | Amidinotransferase family protein                       | 40 down-regulated            |
| Contig16579 | Clan family ubiquitin hydrolase-like cysteine peptidase | 20 down-regulated            |
| Contig11326 | Bestrophin-3- partial                                   | 19 down-regulated            |
| Contig9477  | Anion bicarbonate porter family member (abts-1)         | 10 down-regulated            |
| Contig16506 | Glucosamine-fructose-6-phosphate aminotransferase       | 5 down-regulated             |
| Contig34744 | Peroxidasin   | 3 down-regulated             |
| Contig11546 | Trehalase family protein                                | 3 down-regulated             |

<sup>a</sup> Tentative annotation is based on the most significant BLAST alignment.

<sup>b</sup> Fold change for each contig is calculated by dividing the abundance of reads for the contig in plant diet condition by the reads for diet-changed condition.



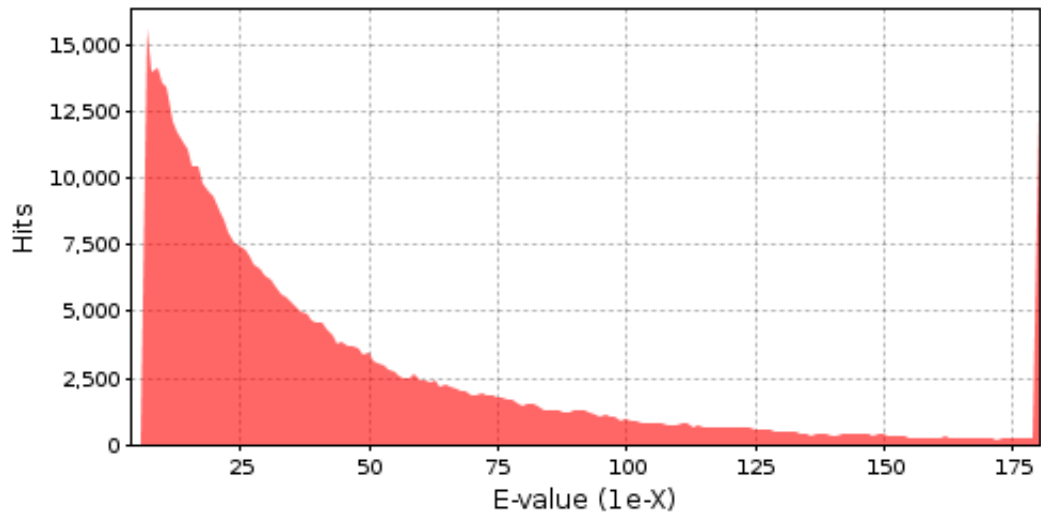


Figure 2.1. E-value distribution of the unigenes with BLAST hits of *Aphelenchoides fragariae* transcriptome. Unigenes were queried against the Non-redundant (nr) database, number of BLAST hits were set to 20. The figure was drawn using Blast2GO.

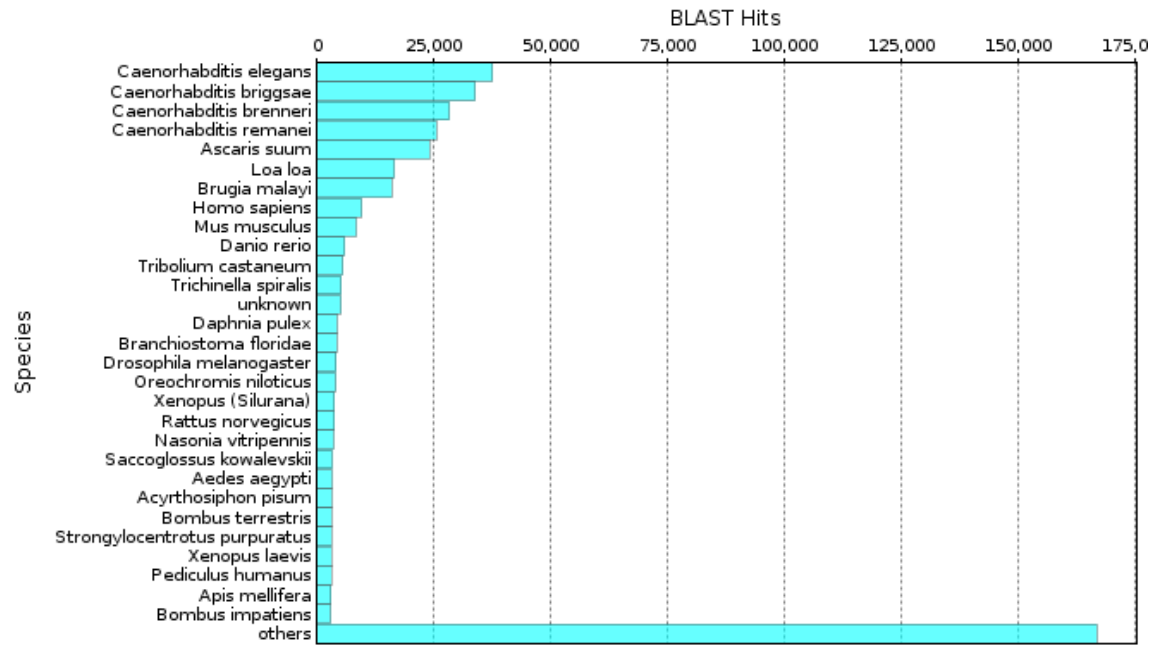


Figure 2.2. Distribution of BLAST hits of *Aphelenchoides fragariae* unigenes compared to species represented in the Non-redundant (nr) database. The number of BLAST hits was set to 20. The figure was drawn using Blast2GO.

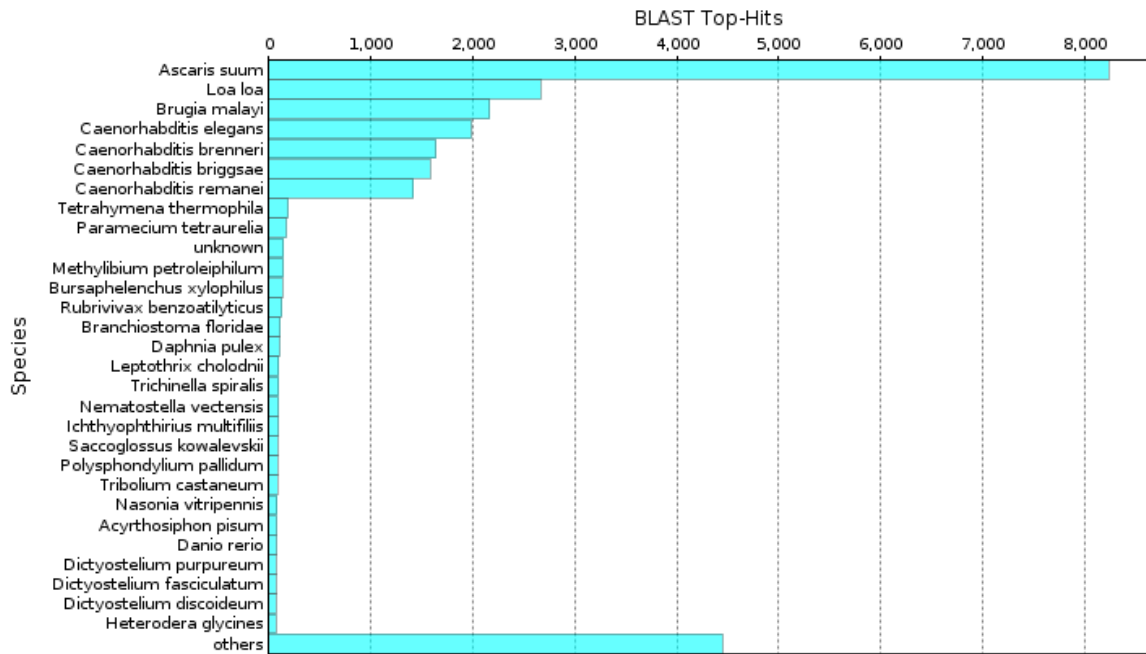


Figure 2.3. Distribution of top hits of *Aphelenchoides fragariae* unigenes compared to species represented in the Non-redundant (nr) database. The figure was drawn using Blast2GO.

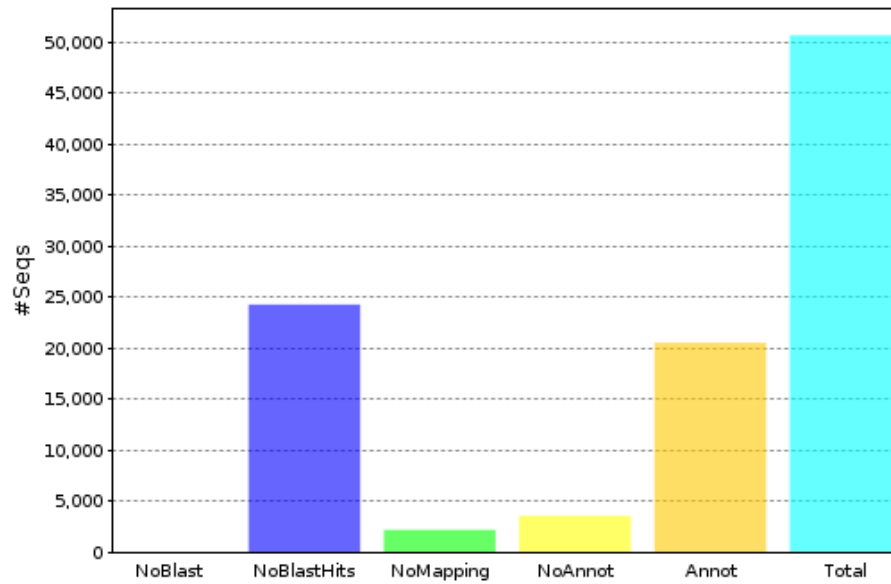


Figure 2.4. BLAST, mapping, and annotation results for a total of 50,686

*Aphelenchoides fragariae* unigenes. Mapping is the process of retrieving Gene Ontology (GO) terms associated to the hits obtained after a BLAST search. The figure was drawn using Blast2GO.

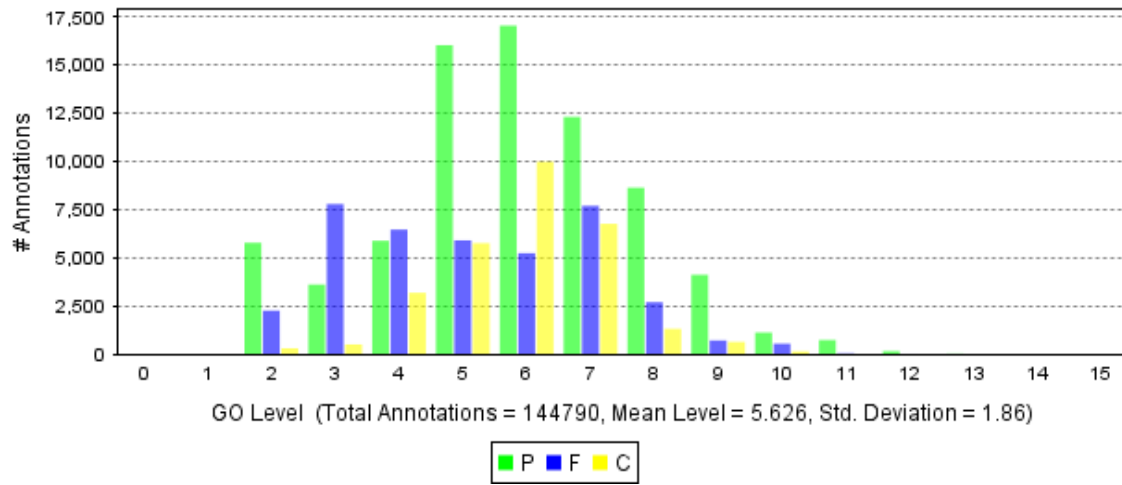


Figure 2.5. Gene Ontogeny (GO)-level distribution of the annotated *Aphelenchoides fragariae* unigenes. P: biological process, F: molecular function, C: cellular component.

The figure was drawn using Blast2GO.

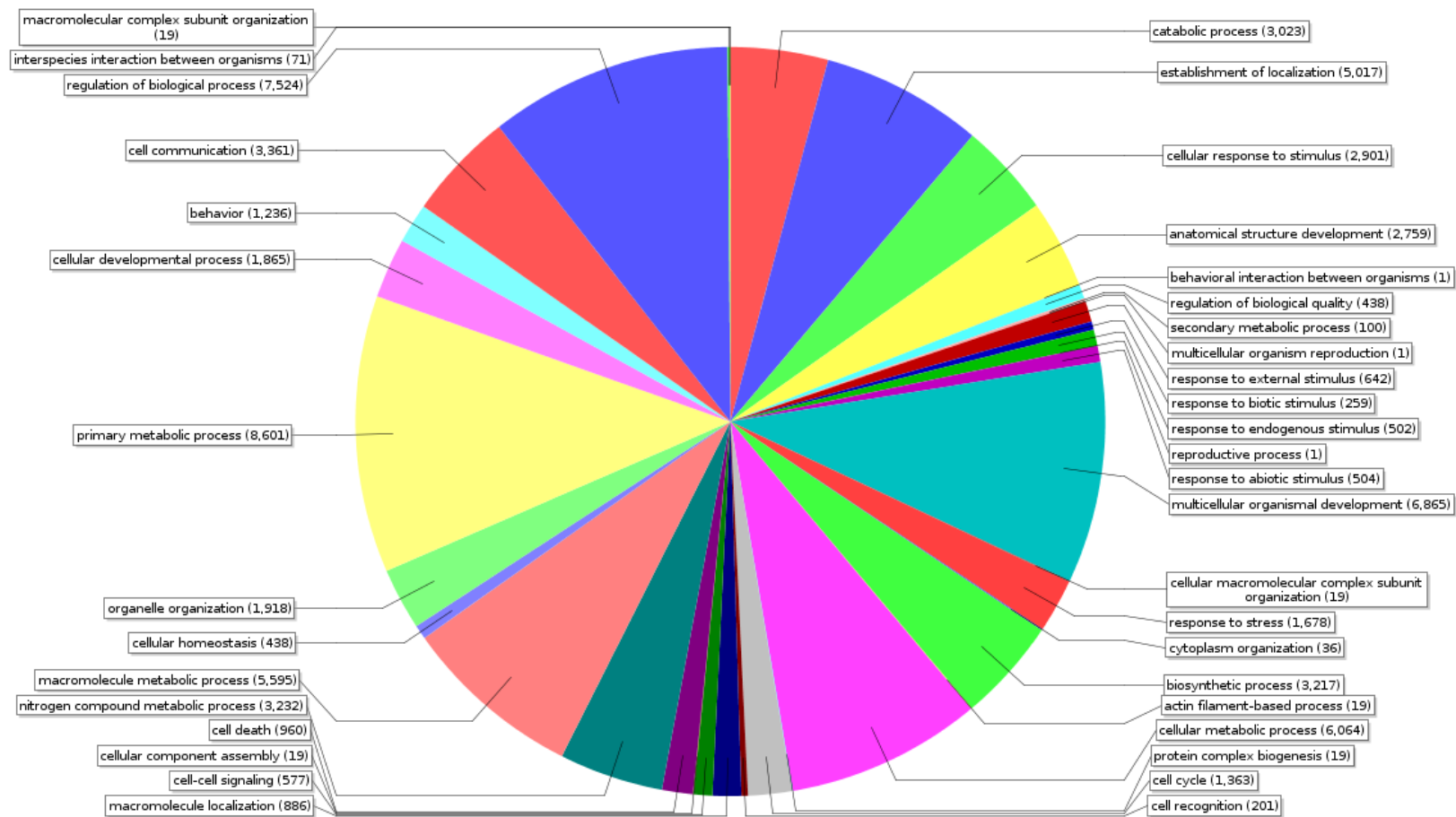


Figure 2.6. Representation of gene ontology (GO) mapping for the *Aphelenchoides fragariae* unigenes in biological processes.

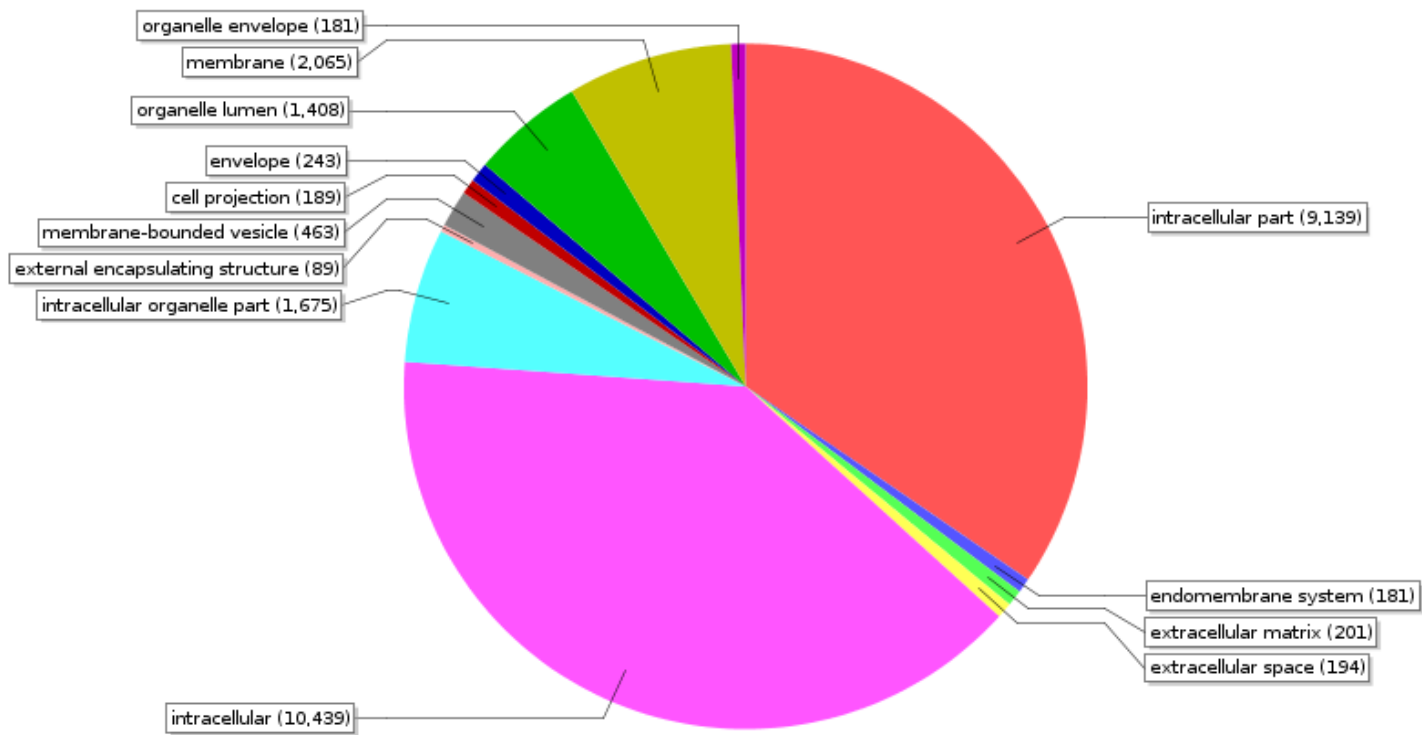


Figure 2.7. Representation of gene ontology (GO) mapping for the *Aphelenchoides fragariae* unigenes in cellular components.

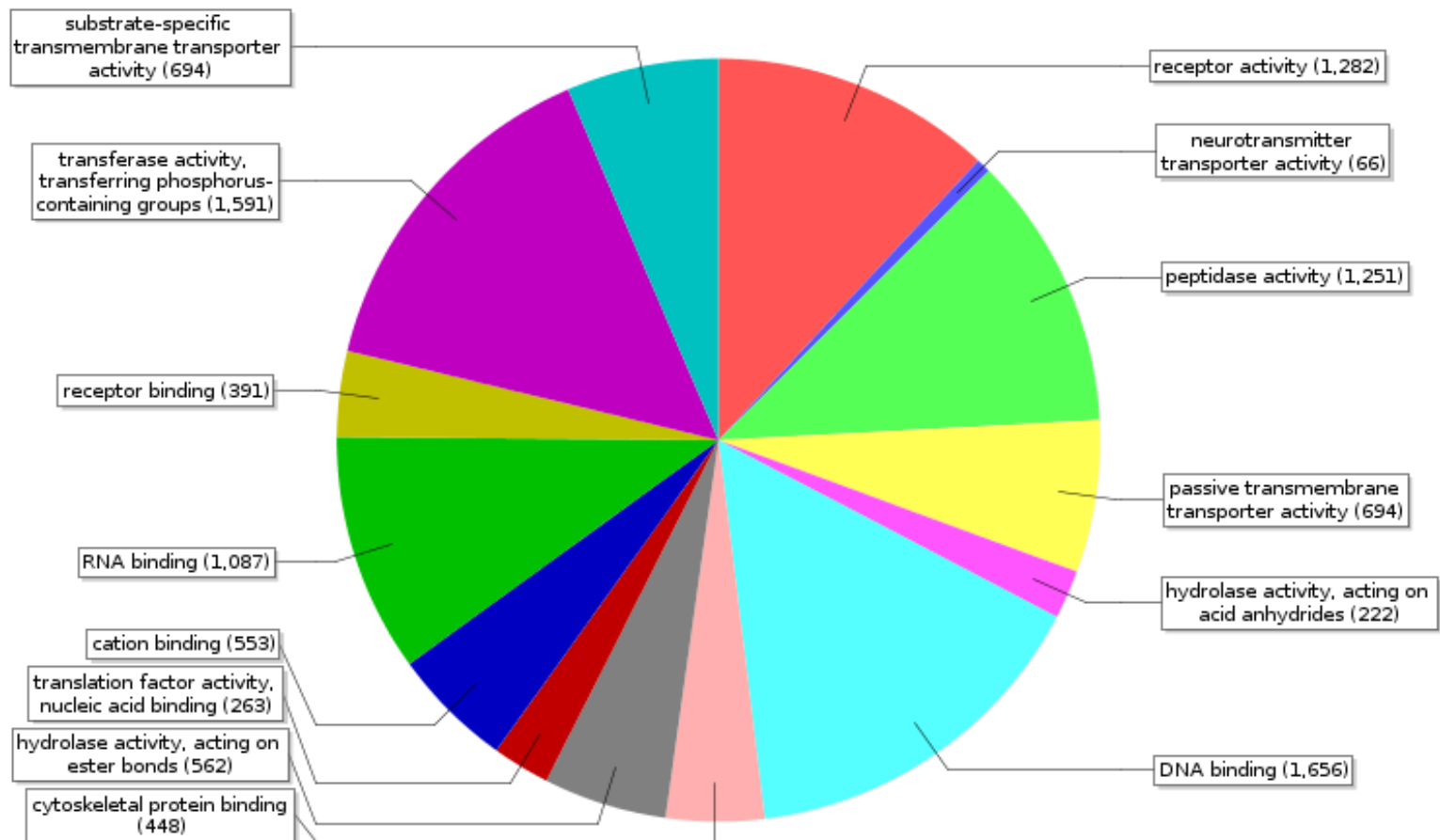


Figure 2.8. Representation of gene ontology (GO) mapping for the *Aphelenchoides fragariae* unigenes in molecular functions.



## CHAPTER THREE

### DIFFERENTIAL EXPRESSION OF A BETA-1,4-ENDOGLUCANASE INDUCED BY

#### DIET CHANGE IN THE FOLIAR NEMATODE

##### *APHELENCHOIDES FRAGARIAE*

The foliar nematode *Aphelenchoides fragariae* is an endo- and ectoparasite of above ground parts of many plants. The nematode enters the leaf tissue through stomata or wounds, feeds on mesophyll cells (Sanwal, 1959), and causes characteristic vein-delimited lesions. The first line of defense that invading nematodes confront upon entering the leaf is the cell wall, of which cellulose is a primary component. In order to access the nutrient-rich cytoplasm, nematodes produce cell-wall-degrading enzymes to weaken the wall. The esophageal gland cells of plant-parasitic nematodes have evolved to secrete cellulases, which are injected via the stylet into the plant tissue (Davis *et al.*, 2004). Over the last decade, many PPN cell-wall-degrading enzymes have been identified and studied. Endo-1,4- $\beta$  glucanases (E.C 3.2.1.4), which degrade the  $\beta$ -1,4-linkage of cellulose, are some of the better characterized. The first glycosyl hydrolase family 5 (GHF5) genes reported in the animal kingdom were *Gr-eng-1*, *Gr-eng-2*, *Hg-eng-1* and *Hg-eng-2* from *Globodera rostochiensis* and *Heterodera glycines*, respectively (Smant *et al.*, 1998). Other endoglucanases have been identified in root-knot nematode *Meloidogyne incognita* (Rosso *et al.*, 1999), stem and bulb nematode *Ditylenchus africanus*, lesion nematode *Pratylenchus coffeae* (Kyndt *et al.*, 2008), and burrowing

nematode *Radopholus similis* (Haegeman *et al.*, 2008). Most of the GHF5 genes studied on PPNs correspond to obligate parasites of the superfamily Tylenchoidea.

Cellulases GHF 45 (Kikuchi *et al.*, 2004), GHF 16 (Kikuchi *et al.*, 2005) and GHF 5 (Kikuchi *et al.*, 2004) have been identified in pine wood nematode *Bursaphelenchus xylophilus*, which demonstrates the involvement of cellulases other than GHF 5 in facultative feeding strategies. *Aphelenchoides fragariae* is also a facultative plant parasite (i.e. having the ability to feed on both plants and fungi) of the same taxonomic family as *B. xylophilus*. Plant parasites in this family feed mainly on aerial parts of plants, and include pine wood nematode *B. xylophilus*, chrysanthemum foliar nematode *Aphelenchoides ritzemabosi*, rice white tip nematode *Aphelenchoides besseyi*, and other economically important species.

Isolates of *B. xylophilus* that are more vigorous in the mycophagous phase have shown reduced virulence on pine (Wingfield, 1987). Likewise, we have observed differences in virulence between *A. fragariae* isolates reared on fungi vs. plants (referred to Chapter one). The mechanisms associated with feeding preferences and the factors that govern the coordination of the mycophagous and phytophagous behaviors in the life cycle of foliar nematodes have not been elucidated. It is likely that enzymatic changes regulated by gene expression underlie these feeding preferences and diet changes. Differential expression of  $\beta$ -1,4-endoglucanase genes has been shown on different developmental stages of PPNs (Goellner *et al.*, 2000; Haegeman *et al.*, 2008), but no research has been published on the differential expression of nematode cellulases under different diets.

The objectives of this study were to identify and characterize a  $\beta$ -1,4-endoglucanase in *A. fragariae*, determine the expression levels of this enzyme under plant and fungus diets, and examine phenotypic differences associated with different diets.

## **Materials and Methods**

***Nematode materials.*** The nematodes used in this study were identified by morphology (Siddiqi, 1975) as *A. fragariae* and labeled according to their diet: plant, fungus, and diet-changed. The Fungus diet nematodes are part of the Clemson University Nematode Collection and have been cultured *in vitro* on *Cylindrocladium* sp. grown in potato dextrose agar (PDA, HiMedia laboratories, India) under laboratory conditions for 25 years. The origin of the isolate is uncertain. The Plant diet nematodes were isolated from infected hosta plants in the South Carolina Botanical Garden, identified by morphology, and cultured on hostas in the field and the greenhouse since 2009. The Diet-changed nematodes were obtained by transferring approximately 1,000 individuals from the Plant diet nematodes to a fungus culture. For this, the nematodes were washed three times with 0.5% streptomycin sulfate (MP Biomedicals, OH, USA), washed once with 0.5% chlorhexidine diacetate hydrate (Acros Organic, NJ, USA), and rinsed in sterilized tap water twice. Surface-sterilized nematodes were transferred to the *Cylindrocladium* sp. culture on PDA. The cultures were kept in an incubator at  $25\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$  for five generations, assuming a life cycle of 12 days. Prior to measurement and nucleic acid extraction, all nematodes were extracted from their cultures using the Baermann funnel technique (Baermann, 1917).

***DNA extraction and Degenerate PCR.*** Genomic DNA of Plant diet nematodes and Fungus diet nematodes was extracted from 20 individuals using Sigma Extract-N-Amp kit (XNAT2) (Sigma, St. Louis, MO). The manufacturer's protocol was modified by reducing all volumes to one fourth of the recommended amounts. Twenty nematodes were placed into a 0.2 ml centrifuge tube containing 25  $\mu$ l of the kit's Extraction Solution. The nematodes were crushed using the tip of a <10  $\mu$ l pipette tip, followed by addition of 7  $\mu$ l of the kit's Tissue Prep solution to the tube. The tube was then vortexed followed by a brief centrifugation to collect contents. The mix was then incubated at 55  $^{\circ}$ C for 10 min, followed by incubation at 95  $^{\circ}$ C for 3 min. Next, 25  $\mu$ l of the kit's Neutralization Solution was added to each tube and extracted DNA was used for PCR or stored at -20  $^{\circ}$ C. Amplification by PCR was conducted using 2  $\mu$ l of these DNA extracts and degenerate primers for nematode endoglucanases designed by Rosso *et al.*, (1999): Eng1: 5'-TAY GTI ATH GTI GAY TGG CA-3', Eng2: 5'-GTI CCR TAY TCI GTI ACR AA-3'. Reactions of 25  $\mu$ l contained 1X ReadyMix Taq PCR Reaction Mix (Sigma-Aldrich, St. Louis, USA): 50 mM of KCl, 1.5 mM of MgCl<sub>2</sub>, 0.2 mM of dNTP, 0.2  $\mu$ M of each primer, and 1.5 units of Taq DNA polymerase. PCR cycling parameters were initial denaturation of 94  $^{\circ}$ C for 2 min followed by 35 cycles of 94  $^{\circ}$ C for 30 s, 47  $^{\circ}$ C for 1 min, and 72  $^{\circ}$ C for 2 min, and a final extension at 72  $^{\circ}$ C for 5 min. PCR products were visualized on a 2% agarose gel stained with ethidium bromide. Amplicons were recovered from the gel using MinElute Gel Extraction Kit (Qiagen, MD, USA) and sequenced at Clemson University Genomic Institute. The sequence of the amplicon was

verified to be an endoglucanase using blastn (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Gene-specific primers were designed based on the Plant diet nematodes amplicon sequence using Primer3 (Rozen and Skaletsky , 2000) to amplify a product of 186 bp.

***RNA extraction and quantitative reverse transcription PCR (qRT-PCR).*** Total RNA of 5,000 nematodes from each diet was extracted using RNeasy Mini kit (Qiagen, MD, USA). Total RNAs were treated with DNase I (Promega, Madison, USA) at 37 °C for 30 min and then at 65 °C for 10 min. Quantitative reverse transcription PCR reactions were set up in 25 µl following the instructions of the QuantiTect SYBR Green RT-PCR kit (Qiagen, MD, USA) on a Stratagene Mx3000P QRT-PCR system, using 0.5 µl of the total RNA from the nematodes cultured on the three different diets as templates.

Quantitative reverse transcription PCR parameters included initial reverse transcription at 50 °C for 30 min, denaturation at 95 °C for 15 min followed by 40 cycles of 94 °C for 15 s, 50 °C for 30 s, and 72 °C for 30 s (at the end of the this step, fluorescence data were collected). The dissociation curve (melting curve) was included at the end of the program from 55 °C to 95 °C. A portion of the 28S ribosomal RNA gene (154 bp) was included for all samples as reference for normalization of products. The primers used in the qRT-PCR were Eng-F: 5'-GTT GAC GTT GCT GCT CAA AA-3', Eng-R: 5'- CCG TAT TCG GTG ACG AAG AT-3'; and 28S-F: 5'-AGT GGG ACA CTT GGT GTC TGT GA-3', 28S-R: 5'-TCT GAC TTC GTC CTG TTC GGG CA-3'. Four replicates were included for each nematode diet. The standard curve method was used to quantify differences in expression. A dilution series for the Plant diet nematode total RNA was

used to create a standard curve for both the 28S gene and *Afr-eng*. Threshold (Ct) values from each sample were converted to the relative copy number based on the standard curve, and the *Afr-eng* expression fold changes between diets were calculated. The 28S fold change was calculated the same way. The fold change of the endoglucanase gene corrected for the housekeeping gene was obtained by dividing the endoglucanase fold change by the 28S gene fold change. All amplicons were treated with ExoSAP-IT (Affymetrix, Santa Clara, CA) and sequenced at Clemson University Genomic Institute.

***Detection of Afr-eng from genomic DNA of single nematodes.*** Sixteen single nematodes from each diet were hand-picked into individual PCR tubes. Genomic DNA extraction was conducted as described above, following manufacturer's instructions for the Extract-N-Amp Tissue PCR Kit (Sigma-Aldrich, St. Louis, USA) and modifying to use one tenth of the recommended volumes. Reactions were set as described above, using 2 µl of the DNA extract from each individual and primers Eng-F and Eng-R for amplification of *Afr-eng-1*, and 28S-F and 28S-R for amplification of 28S ribosomal gene as control for DNA extraction and PCR amplification. The PCR cycling parameters were an initial denaturation at 94 °C for 2 mins, followed by 45 cycles of 94 °C for 30 s, 51 °C 1 min and 72 °C for 2 mins, and a final extension of 72 °C for 5 mins. Products were visualized on a 2% agarose gel stained with ethidium bromide. The percentage of individuals producing *Afr-eng-1* positive amplification was calculated.

***Effect of diet on nematode body size.*** Twenty females and twenty males of each diet were arbitrarily picked and mounted on microscope slides. A photo of each nematode was taken via ProgRes C5 (Jenoptik, Germany) connected to an Olympus BX 60 (Olympus, Japan) compound microscope. The body length and body width of each individual were measured using i-Solution (Focus Precision, Victoria, MN). Data on body length and body width for each diet were analyzed by one-way ANOVA with JMP 9 (SAS Institute, Cary, NC). Significant differences in the mean body length and body width between nematode diets were determined by Tukey HSD test at  $P < 0.05$ . The plant and fungus diet nematodes had been cultured for more than 100 generations on their respective diets before measurement.

***Afr-eng sequence analysis.*** *Afr-eng* gene sequences were obtained from an Illumina RNA-seq project of the foliar nematode carried out in our laboratory. The transcriptome was obtained from a mixture of life stages and diets. We established a local database with contigs assembled from 100 bp Illumina reads and blasted the *Afr-eng* partial sequence amplified in the qRT-PCR described above with tblastx algorithms. Three contigs showed high similarity ( $E = 2e^{-120}$ ,  $E = 2e^{-38}$  and  $E = e^{-32}$ , respectively) to the *Afr-eng* partial sequence. The contigs were named *Afr-eng-1*, *Afr-eng-2*, and *Afr-eng-3* and were submitted to Genbank (accession numbers JN851728, JN851727, and JQ768418, respectively). Sequences *Afr-eng-2*, and *Afr-eng-3* were annotated as partial. The open reading frames (ORF) of all three contigs were located with ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). The predicted proteins were queried with

blastp to find homologous proteins from other PPN. The signal peptide was predicted using SignalP 4.0 (Petersen *et al.*, 2011). The protein was searched through Conserved Domains database of NCBI (Marchler-Bauer *et al.*, 2011) to find the domain and protein family. Two  $\beta$ -1,4-endoglucanase proteins, Pp-ENG (BAB68522) from *Pratylenchus penetrans* and Rs-ENG (ABV54448) from *R. similis*, were aligned with Afr-ENG-1, Afr-ENG-2 and Afr-ENG-3 using ClustalW2 (Larkin *et al.*, 2007) and displayed in Jalview (Waterhouse *et al.*, 2009). Degenerate primer flanking regions were located in the aligned protein sequences.

Endoglucanase sequences of PPNs were selected from Genbank and aligned using ClustalW2 (Larkin *et al.*, 2007). A maximum likelihood phylogenetic tree was constructed in PhyML using LG amino acid substitution model and the SPR method of topology estimation (Guindon *et al.*, 2010). The tree was displayed using Interactive Tree Of Life v2 (Letunic and Bork, 2011). The reliability of the tree was assessed using 100 bootstrap replicates. The proteins used for the phylogenetic analysis and their corresponding GenBank Accession numbers are: Mi-ENG-1 (AAK21882), Mi-ENG-2 (AAK21883), Mi-ENG-3 (AAR37374), Hg-ENG-1 (AAC15707), Hg-ENG-2 (AAC15708), Hg-ENG-3 (AAC33860), Hg-ENG-4 (AAP88024), Hg-ENG-5 (AAP97436), Hs-ENG-1 (CAC12958), Hs-ENG-2 (CAC12959), Pp-Eng-1 (BAB68522), Pp-Eng-2 (BAB68523), Dd-ENG-1 (ACJ60676), Dd-ENG-2 (ACP20205), Da-ENG-1 (ABY52965), Bx-ENG-1 (AB179544.1), Bx-ENG-2 (AB179545.1), Bx-ENG-3 (AB179546.1), Aav-ENG-1 (AB495300.1), Aav-ENG-2 (AB495302.1), Rs-ENG-1



(ACB38289), Rs-ENG-2 (ABV54448), Rs-ENG-3 (ABV54449), and outgroup *Trichoderma* sp (M15665).

## Results

**Identification of *Afr-eng*.** No amplicons were detected in the Fungus diet nematodes using the degenerate primers, whereas one clear amplicon (450 bp) was detected in the Plant diet nematodes. Blastn results showed high similarity between this amplicon and *Meloidogyne hapla*  $\beta$ -1,4-endoglucanase, other PPN  $\beta$ -1,4-endoglucanases, and a bacterial cellulase from *Erwinia chrysanthemi* (Table 3.1).

**Differential expression of *Afr-eng* induced by diet change.** The standard curves of 28S gene and *Afr-eng* had  $r^2$  values of 0.999 and 0.997, respectively. The 28S ribosomal gene was detected in all three diets and the dissociation curves for this amplicon were identical. Amplicons of *Afr-eng* with identical dissociation curves were detected on the Plant diet nematodes (average Ct of 22.05) and the Diet-changed nematodes (average Ct of 32.73, Figure 3.1). No amplicon was detected for the Fungus diet nematodes. Relative copy numbers were calculated based on the standard curves (28S,  $y = -3.827\log x + 1.41$  and *Afr-eng*,  $y = -2.697\log x + 20.42$ ). Expression of *Afr-eng* was 1,812-fold higher in the Plant diet nematodes than in the Diet-changed nematodes after correcting with the fold change of the housekeeping gene.

***Detection of Afr-eng-1 in genomic DNA from single nematodes.*** The frequency of detection of *Afr-eng-1* in individuals was different among the three diets (Figure 3.2A). The gene was readily detected in 75% (12 of 16) of the individuals from the plant diet by amplifying a 186-bp portion of the gene. However, in the Diet-changed nematodes the gene was detected in only 37.5% (6 of 16) of the individuals, and four of these six amplicons were 40 bp shorter (146 bp). The alignment of the short and long amplicons (Figure 3.2B) shows the 40 bp intronic sequence responsible for the band shift on the gels. The gene was not amplified in any (0 of 16) of the individuals from the fungus diet.

***Effect of diet change on body size.*** There were significant differences ( $P < 0.05$ ) between the Plant diet nematodes and the Fungus diet nematodes in the body length and body width of females and males (Figure 3.3). The females of the Plant diet nematodes were longer (average 690  $\mu\text{m}$ ) and thinner (average 17.0  $\mu\text{m}$ ) than females of the Fungus diet nematodes. The Fungus diet females were an average of 609  $\mu\text{m}$  long and 20  $\mu\text{m}$  wide, and the Diet-change females were an average of 643  $\mu\text{m}$  long and 22.3  $\mu\text{m}$  wide. There were no differences in the body length for the males, but males from the Plant diet were significantly ( $P < 0.05$ ) thinner (average 13.99  $\mu\text{m}$ ) than the Fungus diet males (average 20.2  $\mu\text{m}$ ) and the Diet-changed males (19.9  $\mu\text{m}$  average). There were no differences ( $P < 0.05$ ) in the body measurements for males or females between the Fungus diet and the Diet-changed. The Diet-changed nematodes have the same origin as the Plant diet nematodes, but after five generations of feeding on the fungus, their

appearance resembles that of the Fungus diet nematodes and is different from the parent population.

**Characterization *Afr-eng-1*.** Of the three putative endoglucanases identified (*Afr-eng-1*, *Afr-eng-2*, and *Afr-eng-3*), only *Afr-eng-1* aligned perfectly with the partial *Afr-eng* sequence amplified via qRT-PCR (Figure 3.4), confirming this is the sequence being amplified and differentially expressed. When mapping the short reads generated by Illumina to *Afr-eng-1*, the abundance of reads for the “Plant diet nematodes” was 17,714, while the abundance of reads for the “Diet-changed nematodes” was 1.57, based on the calculation of RSEM (<http://deweylab.biostat.wisc.edu/rsem/>). *Afr-eng-1* encodes for a 320 aa protein, and its signal peptide (1-17 aa), GHF 5 domain (33-278 aa), and predicted active sites (positions 152 and 239) are shown in Figure 3.5.

BLAST results indicate that all three putative proteins are homologous to other PPN beta-1,4-endoglucanases: *R. similis* Rs-ENG (ABV54446;  $E=1e^{-154}$ ), *P. coffeae* Pc-ENG (ABX79356;  $E=4e^{-150}$ ), *P. penetrans* Pp-ENG (BAB68522;  $E=3e^{-148}$ ), *M. incognita* Mi-ENG (No. AAD45868.1;  $E=4e^{-135}$ ). The alignment of two representative sequences from other plant nematodes with the three predicted endoglucanases is shown in Figure 3.6.

**Phylogenetic analysis.** A maximum likelihood tree, condensed with cut off value of 50% from the bootstrap consensus tree, is shown in Figure 3.7. *Afr-ENG-1*, *Afr-ENG-2* and *Afr-ENG-3* form a tight cluster and were placed in a large clade with *Pratylenchus*, *Meloidogyne*, *Aphelenchus*, *Heterodera*, *Radopholus* and *Ditylenchus* endoglucanases.

The *B. xylophilus* beta-1,4-endoglucanases: Bx-ENG-1 and Bx-ENG-2 formed a clade separate from all other PPN beta-1,4-endoglucanases examined in this study.

## Discussion

Plant-parasitic nematodes require anatomical specializations (e.g. the stylet) and an arsenal of gene products to accomplish plant parasitism. Several of these genes are involved in cell-wall degradation, necessary for penetration and migration through the plant tissue. A number of cellulases have been identified as essential to plant parasitism in PPNs, mostly in sedentary and root parasitic nematodes. Only a few non-root PPN cellulases have been studied, including endoglucanases from *D. africanus* (Kyndt *et al.*, 2008) and *B. xylophilus* (Kikuchi *et al.*, 2004). Different glycosyl hydrolase family proteins (GHF 16 and GHF 45) were identified in *B. xylophilus* (Kikuchi *et al.*, 2004; 2005). As a closely related species to *B. xylophilus*, *A. fragariae* shares many similarities with *B. xylophilus*, especially morphological features and facultative plant parasitism. In this work, we report and characterize the first cellulase gene identified in the genus *Aphelenchoides*. Interestingly, based on local alignment results, *Afr-eng* shares more similarities with endoglucanases from *Meloidogyne* spp., *Radopholus* spp. and *Pratylenchus* spp. than with those from *Bursaphelenchus* spp. However, if we consider habitat and niche, the habitat of *B. xylophilus* (wood tissue) would require a different set of adaptations and could explain why the foliar habit may be closer to the root habit in terms of the necessary cellulases. Cellulases from families other than GHF 5 have not yet been identified from *Aphelenchoides* spp., but we would expect the facultative feeding

strategies of *A. fragariae* to render other enzymes necessary to degrade the fungal cell wall. For example, Kikuchi *et al.* (2005) hypothesize that endo-1,3-beta-endoglucanase has a role in facultative fungal feeding of *B. xylophilus*, as beta-1,3-glucan is a main component of fungal cell walls.

No genomic PCR amplicons were detected in the Fungus diet nematodes using degenerate primers Eng1 and Eng2. These primers encode amino acid sequences YVIVDWH and FVTEYGT respectively (Rosso *et al.*, 1999) in the catalytic domain, two short amino acid sequences highly conserved in many PPN species. We were able to locate those two amino acid sequences in the predicted Afr-ENG (Fig. 3.6). We also did not detect any amplicon from the Fungus diet nematodes with qRT-PCR. The lower detection in the Diet-changed nematodes and the lack of detection in the Fungus diet nematodes suggest selection against the portion of the population in which this gene is readily detectable. In the original Plant diet nematodes, the frequency of the individuals in which the gene is readily detectable is high (75%) and the amplicon includes a 40 bp intron present in only some of the amplicons of the Diet- changed nematodes. It is possible that alternative splicing of the *Afr-eng-1* is responsible for this amplicon size difference, but further sequencing of each individual is necessary to test this hypothesis. The portion of the population for which the gene cannot be detected by the method we employed increased as the diet shifted to fungus. This could be caused by variation in copy number, polymorphisms, or a reason yet to be determined by further studies.

In other studies,  $\beta$ -1,4-endoglucanases from pinewood nematodes, *B. xylophilus*, have been isolated from cultures maintained on fungi (Kikuchi *et al.*, 2004; Ma *et al.*, 2011).

These nematodes were originally isolated from trees and then maintained on the fungus *Botrytis cinerea* for easy manipulation. It is possible that they had not completely adapted to the fungus diet. Based on the qRT-PCR analysis, we detected a large decrease in *Afr-eng-1* expression in the Diet-changed nematodes compared to the Plant diet nematodes. Additionally, fewer individuals in the population were detected bearing *Afr-eng-1* after the diet shift from plant to the fungus, which might suggest selection acting on the population (Sved, 1968). Further population genetic analysis is needed to clearly conclude that large decreasing of *Afr-eng-1* is due to population selection or individual gene expression change.

Some researchers have proposed that PPN endoglucanase genes may have been acquired horizontally from bacteria (Jones *et al.*, 2005; Yan *et al.*, 1998), due to the high similarity of the endoglucanases found in PPN and prokaryotes. Nonetheless there is no experimental evidence to support such an origin of PPN endoglucanase genes. It is also possible that the endoglucanases in the PPN are produced by symbionts, as is the case with other nematodes (Sicard *et al.*, 2004), and that the diet change impacts the community composition or population of these symbionts, but this has not been demonstrated yet.

We observed two phenotypic changes associated with diet changes: altered symptom severity and altered body size. In greenhouse studies (refer to Chapter one), using four different hosta cultivars, we observed differences ( $P < 0.05$ ) in the severity of symptoms caused by foliar nematodes maintained on the fungus (leaf area affected ranged from 1.21% to 4.13%) and by those maintained on the plants (leaf area affected ranged from

4.17% to 25.95%). Nematodes maintained on plants for more than 100 generations also had higher reproduction on the hosta cultivars evaluated. These observations are consistent with the expression profiles of *Afr-eng* in the Plant diet nematodes and Fungus diet nematodes and indicate the potential role of this enzyme in penetration and migration. Availability of nutrients, especially of nitrogen, in plants is a limiting factor for herbivores (Mattson, 1980). Plant parasitic nematode body size tends to decrease at lower nutrient availability (Verschoor *et al.*, 2001). The different sources and availability of nitrogen in fungal and plant diets may explain the changes in body size. It is difficult to determine which diet source provides higher nutrients because one diet produced longer and thinner individuals, while the other produced shorter, but wider adults. Body size, in this case, could be more likely associated with movement and feeding behavior. Feeding on plants, especially as an endoparasite, arguably would benefit from a thinner figure and would require more energy than ectoparasitic feeding on fungi.

There is evidence of endoglucanases acting as parasitism genes during the penetration and migration events of the infection process. Differential expression of endoglucanases has been observed in different life stages of PPNs: low expression of endoglucanases has been found in J3, J4 and sedentary females of reniform nematodes (Wubben *et al.*, 2010) and in all non-infective stages of root-knot and cyst nematodes (Goellner *et al.*, 2000; Haegeman *et al.*, 2008). In the case of foliar nematodes, all the life stages are infective and all the developmental stages move and feed on plant tissue. We have found that expression levels of endoglucanases are relevant to symptom severity.

Endoglucanases play an important role in the host-parasite relationships of foliar nematodes and plants. Our studies suggest that they may be significant pathogenicity factors. More studies are necessary to identify the involvement of different endoglucanases and how they participate in regulating the phytophagous and mycophagous behaviors of these nematodes.



Table 3.1. Homologs of the partial *Afr-eng* sequence identified in *Aphelenchoides fragariae* using degenerate PCR.

| Species                       | Identity | Genes                              | E value              | Accession  |
|-------------------------------|----------|------------------------------------|----------------------|------------|
| <i>Meloidogyne hapla</i>      | 71%      | beta-1,4-endoglucanase             | 1.00E <sup>-15</sup> | AY277718.1 |
| <i>Meloidogyne incognita</i>  | 71%      | beta-1,4-endoglucanase (eng-6)     | 1.00E <sup>-15</sup> | AF323091.1 |
| <i>Ditylenchus africanus</i>  | 70%      | endoglucanase (engdel4) pseudogene | 5.00E <sup>-14</sup> | GU139193.1 |
| <i>Meloidogyne arenaria</i>   | 69%      | beta-1,4-endoglucanase (eng-2)     | 2.00E <sup>-12</sup> | AF323098.1 |
| <i>Meloidogyne javanica</i>   | 69%      | beta-1,4-endoglucanase (eng-1)     | 7.00E <sup>-12</sup> | AF323099.1 |
| <i>Heterodera glycines</i>    | 70%      | beta-1,4-endoglucanase-1 precursor | 9.00E <sup>-11</sup> | AF006052   |
| <i>Ditylenchus destructor</i> | 66%      | beta-1,4-endoglucanase             | 4.00E <sup>-09</sup> | FJ430142.1 |
| <i>Erwinia chrysanthemi</i>   | 66%      | extracellular endoglucanase        | 3.00E <sup>-04</sup> | Y00540.1   |

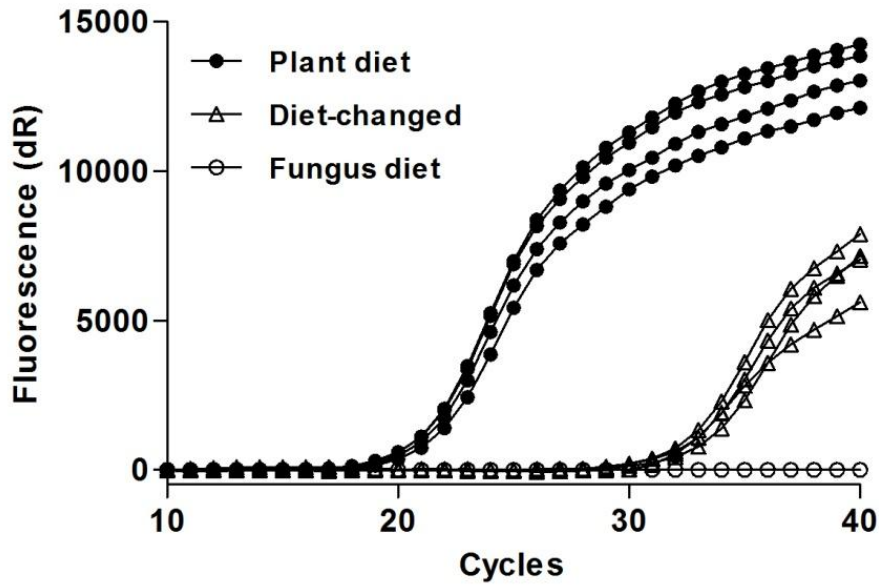
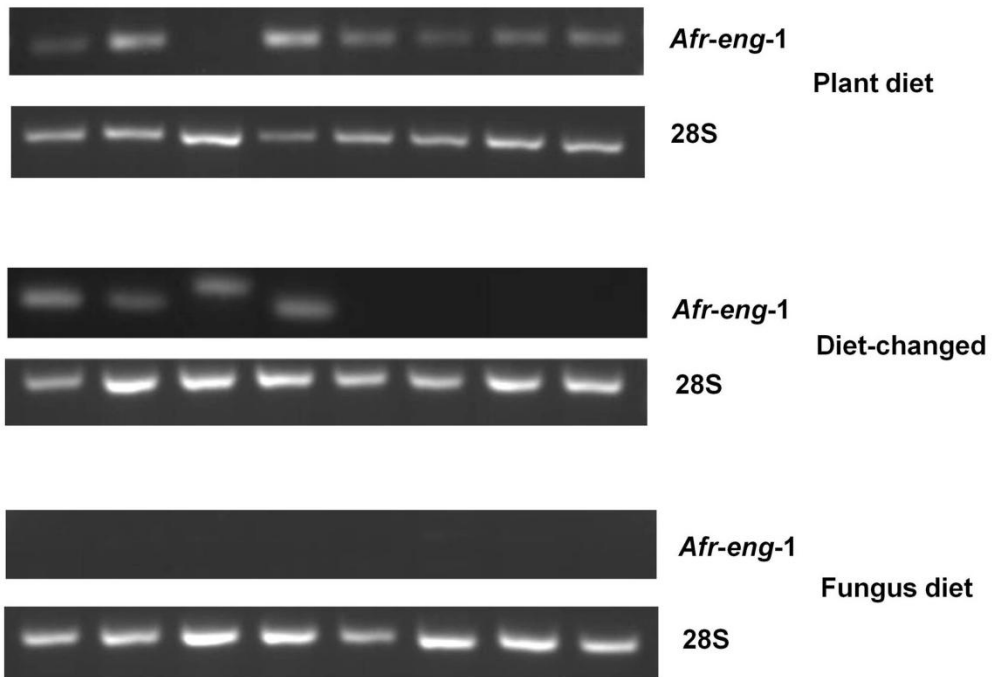


Figure 3.1. Differential expression of *Afr-eng-1* using qRT-PCR of Plant diet foliar nematodes *Aphelenchoides fragariae* and Diet-changed foliar nematodes. No amplification was detected for Fungus diet nematodes. X axis: number of cycles, Y axis: fluorescence units. Each diet was replicated four times.

A



B

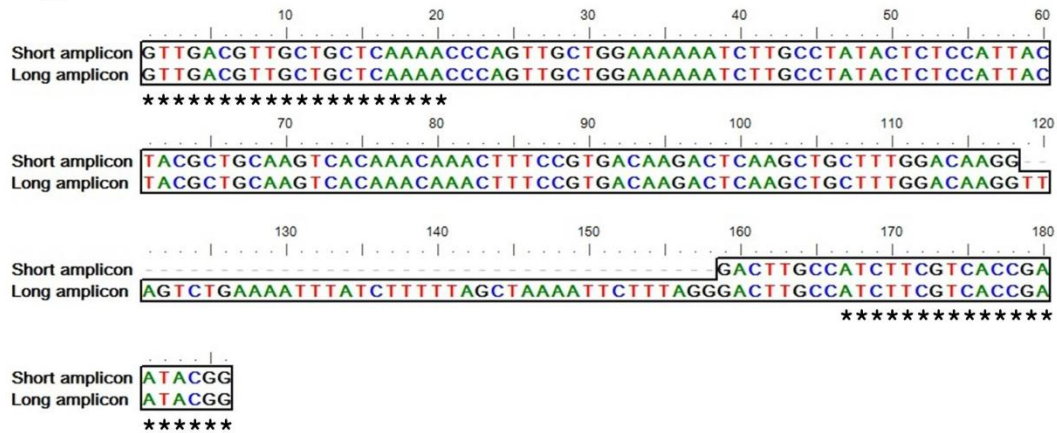


Figure 3.2. A. Detection of *Afr-eng-1* from genomic DNA of single *Aphelenchoides fragariae*. Amplicons from eight individuals from each diet are shown and 28S ribosomal gene is used as control. The two endoglucanase amplicons shown are 146 bp and 186 bp. Plant diet nematodes only showed the long amplicon, Diet-changed

nematodes showed either, and Fungus diet nematodes showed none. B. Alignment of the short and long amplicon, illustrating the intronic 40 bp difference between the two sequences. Primer flanking regions are indicated with asterisks.

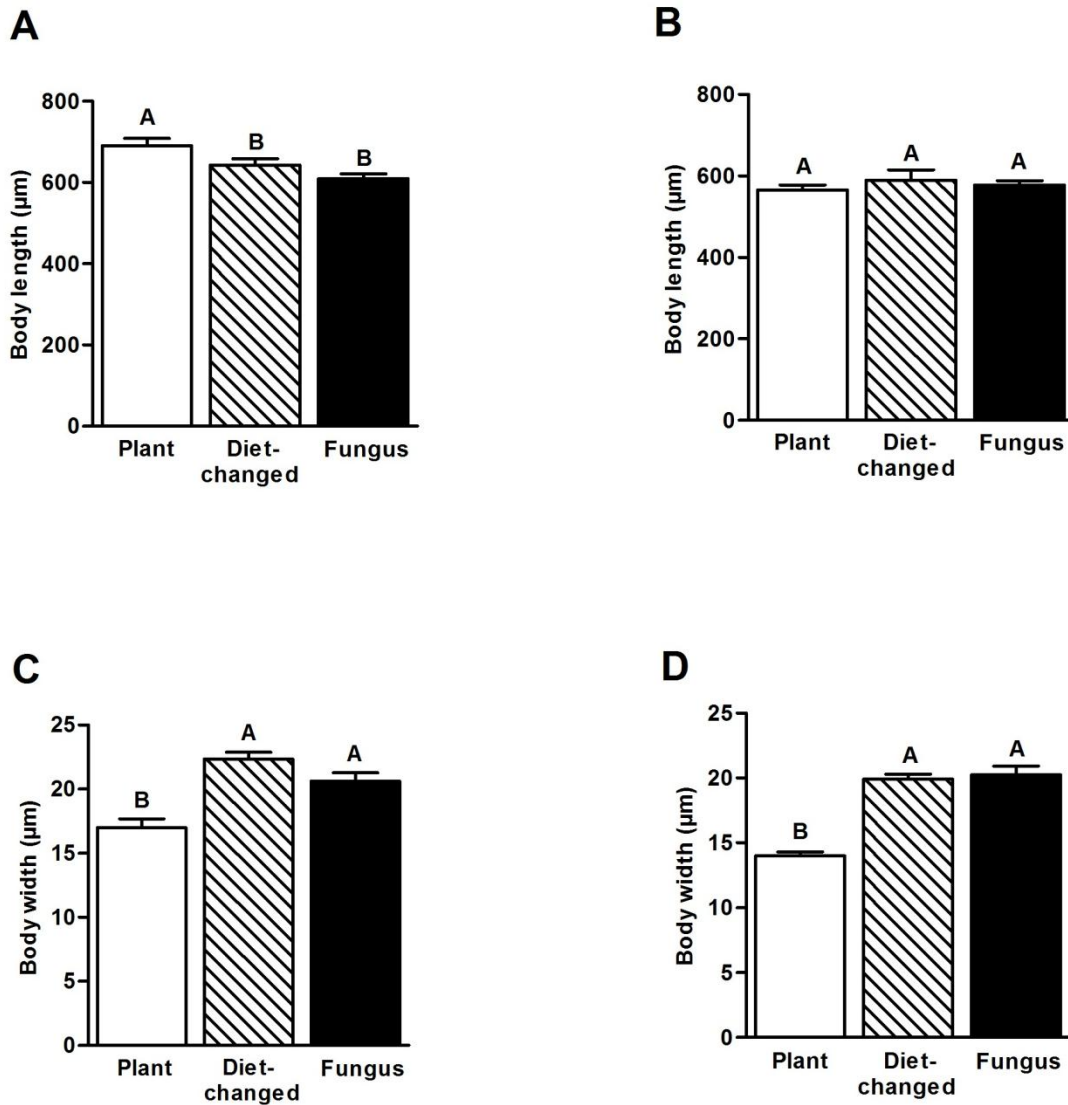
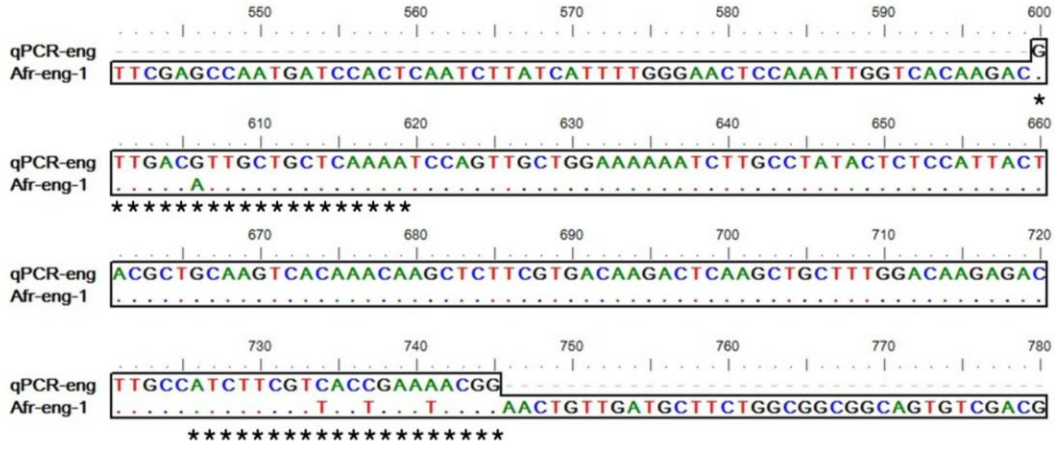
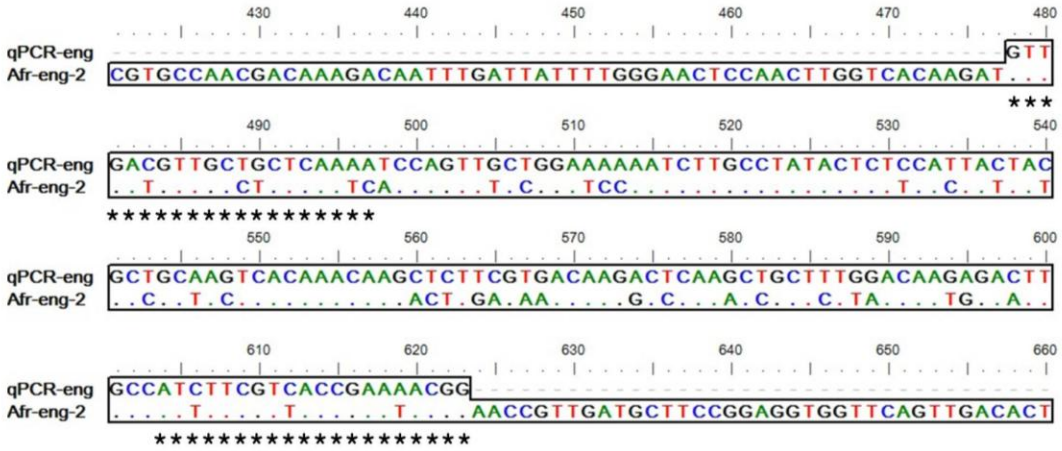


Figure 3.3. Effect of diet on body size measurements of *Aphelenchoides fragariae*. Different letters indicate significant differences ( $P < 0.05$ ) within the same gender according to Tukey HSD test. For each combination of gender and diet, 20 individuals were measured. A. Effect of diet on female body length. B. Effect of diet on male body length. C. Effect of diet on female body width. D. Effect of diet on male body width. Error bars indicate the standard error.

**A**



**B**



**C**

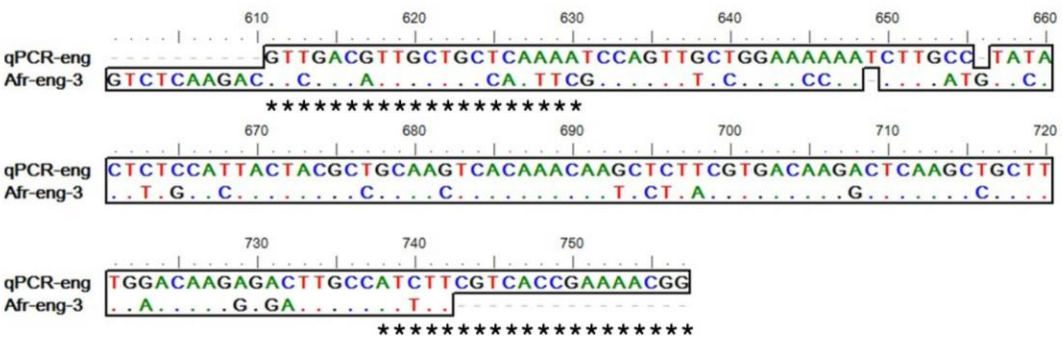
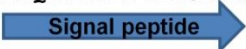




Figure 3.4. Sequence alignment of qRT-PCR partial endoglucanase with *de novo* assembled A. *Afr-eng-1*, B. *Afr-eng-2* and C. *Afr-eng-3* from the transcriptome of *Aphelenchoides fragariae*. Gene specific primers Eng-F and Eng-R designed from product of degenerate PCR were used in the qRT-PCR and were marked as asterisks. The qRT-PCR sequence has the highest similarity with *Afr-eng-1*. The mismatched sequences in the assembled contigs are labeled as nucleotide letters.

MIQVAAVLFFVFIGFANAVAPPYGQLSVKGNKILGADGKPVALHGMSLFWSQWEGGAPFYNKDTVQALKCS 70  

Glycosyl hydrolase family 5

WNANLVRAAMGVVEGGYLTNQATEQGKLEAVVKAIDLGIIYVIIDWHDHHAYQHDPKAVEFFSAMSCKYA 140  
Glycosyl hydrolase family 5


 GVPNIIYEIFNEPLSVSWTSDLVPYHNKVISAIRANDPLNLIILGTPNWSQDVDIAAQNPVAGKNLAYTL 210  
Glycosyl hydrolase family 5


 HYAASHKQALRDKTQAALDKRLAIFVTEYGTVDASGGGSVDVQSSNEWWAFDANKISYANWAI GNKAE 280  
Glycosyl hydrolase family 5

AAAALTPGTTAQQVGDSDLTASGKLVKQKLKSNNGVSC 320

Figure 3.5. Predicted sequence of *Aphelenchoides fragariae* endoglucanase Afr-ENG-1 (Genbank accession No. JN851728). The signal peptide is indicated by the big arrow (position 1 to 17), the glycosyl hydrolase family 5 is highlighted (position 33 to 278) and the predicted active sites are indicated by the small arrows (positions 152 and 239).



```

Afr-ENG-1/1-320 . . . . . MIQVAAVLFVY IGFANAVAPPYQQLSVKGNK I LGADGKPVALHGMSL FWSQWEGEGAPFYNKD TVQAL KCSWN 72
Afr-ENG-2/1-426 . . . . . MSL FWSQWSEGSFAFY NADTVQAL KCSWN 28
Afr-ENG-3/1-236 . . . . . MALLQLAALLSAFVVMTNNAVAPPYQQLSVKGSKLLG SNGQVEVALHGMSL FWSQWSEGSVFY NKD TVQAL KCSWN 74
Pp-Eng/1-455 MTSSSSSMALLVLC LPLQFFLVLAADPPYQQLKVS GSKLVG SNGQAVALHGMSL FWS S FSEGS PFY TADVVKQL KCSWN 81
Rs-Eng/1-319 . . . . . MLSLFL LPL - FFAFASAANPPYQQLSVSGTKLKGSSGQDVALHGMSL FWSNFGEGAPFYNAATVKAL KCKWN 71

Afr-ENG-1/1-320 ANLVRAMGVVEEG - GYL TNQATEQ GKLEAVVKAA IDLGI YVTTIDWH DHHAHQHPDKAVEFFSAMS KKYAGVPNI IYE IFNE 152
Afr-ENG-2/1-426 ANLVRAMGVVEEG - GYL TNPSGEQAKLEAVVEAA ISAGI YVI IDWH DHNAASHTDKAVEFFSAI SKKYAGNPNI IYEVFNE 108
Afr-ENG-3/1-236 ANVVRAMGVVEEG - GYL TNPTAEQAKVETVVQAA IDLGI YVI IDWH DHNAHYHTDKAVQFFSAI SKKYAGVPNVLYE IYNE 154
Pp-Eng/1-455 ANLVRAMGVVEEGSYLSNKQGQMSMVETVIKAAIAEGI YVLVDWH DHNAQNHQSQAIEFFTYIAKTYGNNPHI IYETFNE 162
Rs-Eng/1-319 SNIVRAMGVVEGG - YLNNPSQEQA KLEAVVQAAIAEGI YVI IDWH DHNAQSHQSQA VSF FTAMSKKYGKNTN ILYE IFNE 150

Afr-ENG-1/1-320 PLVSVWTS D LVPYHNKVI SAIRANDP LNI I LGTPNWSQD V D IAAQN PVAG - KNLAYTLHY YAASHKQAL RDKTQAALDKR 232
Afr-ENG-2/1-426 PLQVSWTDV LKPYHTAVVNAIRANDK DNI I LGTPWSQD V D VASQSPVSG - SNLAYTLHY YAATHKQDLR NKAQTALNNG 198
Afr-ENG-3/1-236 PLQVPTD - LKPYHTAVI NAIRANDKNNVI I LGTPWSQD V D IAA NSPVSG - TNLMYTLHY YAATHKQSLRDKAQAALNKG 233
Pp-Eng/1-455 PLQVDWG - VVKPYHVAVVAAIRASDPD NVI VLGTPWSQD V DVAATNPVSG - TNL CYTMHY YAATHKQSLRDKTQAALNKG 241
Rs-Eng/1-319 PLQVDWNSVVKPYAQAVI KAIRANDAKNVI I VGTPTWSQD V DVAANSPI TGQKNIMYTLHY YAATHKQDLR NKLTTAVNKG 231

Afr-ENG-1/1-320 LAI FVTEYGT V DASGGGSVDVQSSNEWWAF L DANKI SYANWA IGNKAEAAAALTPGTTAQQVGD DSRLTASGKLVKQK LKS 313
Afr-ENG-2/1-426 LAI FVTEYGT V DASGGGSVDTQSSNDWWTFLDNNKI SYANWA I DNKAEAAAALTPGTTAQQVGD DSRLTQSGQLVKNK LKG 269
Afr-ENG-3/1-236 LAI FVTEYGT V DASGGGSVDVQSSNEWWAF L DANKI SYANWA IGNKAEAAAALTPGTTAQQVGD DSRLTASGKLVKQK LKS 236
Pp-Eng/1-455 YCV FVTEYGT V SADGNGGMDQASNEWY TFLDNNKI SYANWAVDAKSESSAALNPGTQPSQ I SSDSVL TASGSFVKA K LKS 322
Rs-Eng/1-319 LPV FVTEYGT V TADGNGYVDAAESKTWWNFLDGLKI SYVNWAI DNKDEKSAALTPGTTSSQVGD SRRWTESGKLVQA KLAS 312

Afr-ENG-1/1-320 QNNGVSC . . . . . 320
Afr-ENG-2/1-426 QNNGVSCSGSGSDTTAAQAQT TAAAGQT TAAQQTTATQQQTTTTTKAPGNNDGGSSSEGVSVSVQTVNSWGGGGQYVL 350
Afr-ENG-3/1-236 QNNGVSCSGSPAATTTTKAGATTTKAAATTTKA . . . . . P SGGGGSASVSVSVVAVSTWNGGGGQYKV 383
Pp-Eng/1-455 QNNGVSCSGSPAATTTTKAGATTTKAAATTTKA . . . . . P SGGGGSASVSVSVVAVSTWNGGGGQYKV 383
Rs-Eng/1-319 QKNGVSC . . . . . 319

Afr-ENG-1/1-320 . . . . .
Afr-ENG-2/1-426 AFKNTGSSD ICGADF TLTPSGASTNFWNFQL DSENQYSLPSWVR I SPQGT FQDAGLVVNGDGLPNVSI VSVKKC 426
Afr-ENG-3/1-236 . . . . .
Pp-Eng/1-455 VIKNTGSKAVCKVVF KLSASAASLWN . . . ADPGSGSGPFTTASWIN IPAGQSNE SVGFTVNGSAPS - ASVVSATAC 455
Rs-Eng/1-319 . . . . .

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Figure 3.6. Protein sequence alignment of predicted *Aphelenchoides fragariae* Afr-Eng-1, Afr-ENG-2, Afr-ENG-3 and *Pratylenchus penetrans* beta-1,4-endoglucanase, and *Radopholus similis* beta-1,4-endoglucanase. The degenerate primer flanking regions are boxed.

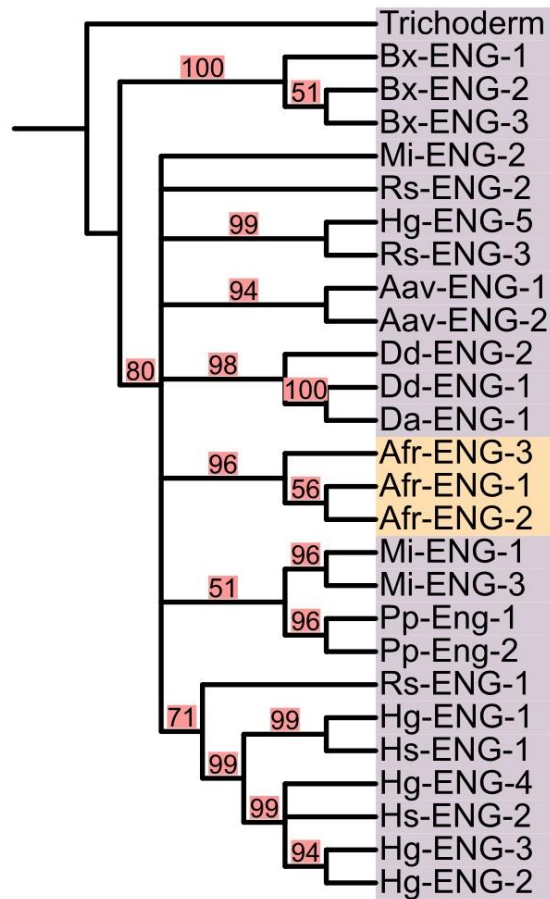


Figure 3.7. Phylogenetic relationships among selected nematode endoglucanases. The tree was constructed by comparisons of the endoglucanases listed in the Materials and Methods, using maximum likelihood of PhyML. The numbers next to the branches indicate the bootstrap value. *Trichoderma* is the outgroup. Abbreviations: Mi: *Meloidogyne incognita*; Pp: *Pratylenchus penetrans*; Afr: *Aphelenchoides fragariae*; Dd: *Ditylenchus destructor*; Da: *Ditylenchus africanus*; Rs: *Radopholus similis*; Hg: *Heterodera glycines*; Hs: *Heterodera schachtii*; Aav: *Aphelenchus avenae*; Bx: *Bursaphelenchus xylophilus*.

## CHAPTER FOUR

### GENES ASSOCIATED TO DESICCATION TOLERANCE OF THE FOLIAR

#### NEMATODE *APHELENCHOIDES FRAGARIAE*

Several nematode species are known to survive under extreme desiccation conditions by entering an anhydrobiotic stage (anhydrobiosis). Viable nematodes have been found from dry desert soil (Frechman *et al.*, 1975) to dry valleys in Antarctic (Treonis and Wall, 2005). Some nematodes can survive under anhydrobiotic stages for many years. It has been reported that the plant parasitic nematode *Ditylenchus dipsaci* survived 23 years under dry storage (Fielding, 1951). During anhydrobiosis, metabolism and aging are suspended (Cooper and Van Gundy, 1971). When anhydrobiotic nematodes are rehydrated, their metabolism and activity resume. However, nematodes do not become active immediately. “Lag phase” is the term denoting the delay of activity (Wharton and Barrett, 1985), which is highly related to the severity of the stress during desiccation (Wharton and Aalders, 1999).

The foliar nematodes *Aphelenchoides fragariae* infect aerial parts of plants, feeding on ornamental plants and other crop plants, causing serious damage in nurseries, greenhouses, and landscapes (Jagdale and Grewal, 2006). The nematodes overwinter in the soil, dormant crowns, and abscised leaves and then migrate to the new leaves in the spring (Jagdale and Grewal, 2006). Desiccation tolerance might be involved with and play an important role in the overwintering survival. In the winter, foliar nematodes may enter anhydrobiosis in the soil and resume activity when free moisture is available. This

ability to survive desiccation might render this pathogen difficult to eliminate even after repeated nematicide applications, but it has not been studied in *A. fragariae*.

Desiccation tolerance has been studied in detail in a few nematode species: stem and bulb nematode *D. dipsaci* and fungivore *Aphelenchus avenae* (Burnell and Tunnacliffe, 2011). The latter belongs to the same family as foliar nematode, *A. fragariae*, and may be a good comparative model for desiccation tolerance. There are some genes identified associated with desiccation and osmotic tolerance in *A. avenae*. Glutaredoxin (GLX) is a member of the redox system of the cells, it is an oxidoreductase that catalyzes the reversible reduction of disulfides of proteins through the cysteine active sites (Ghezzi and Di simplicio, 2009). Glutaredoxin gene showed up-regulation after the desiccation and osmotic tolerance in *A. avenae* (Browne *et al.*, 2004). Trehalose is a disaccharide that has been reported to take up 11% dry weight of nematode under high relative humidity (98%) for 72 h (Browne *et al.*, 2004). The biosynthesis pathways of trehalose have been studied in detail. Trehalose-6-phosphate synthase (TPS) catalyses the conversion of glucose-6-phosphate to trehalose-6-phosphate, which is the first step in trehalose synthesis. The *tps* gene has been identified and is up-regulated under desiccation and cold tolerance in *A. avenae* (Goyal *et al.*, 2005). Other genes associated with desiccation include late embryogenesis abundant (Browne *et al.*, 2002; Ingram and Bartels, 1996), and anhydrin (Browne *et al.*, 2004). The objectives of this study were to 1) test the survival ability of foliar nematodes under different stress conditions, and 2) examine the changes in gene expression associated with desiccation tolerance in *A. fragariae* using *A. avenae* as a comparative model.

## **Materials and Methods**

***Nematode material and stress treatments*** *Aphelenchoides fragariae* propagated on the fungus *Cylindrocladium* sp. grown in potato dextrose agar (PDA) were extracted using a Baermann funnel (Baermann, 1917). Nematodes were washed three times with sterilized tap water. Approximately 50,000 nematodes in 20 ml of sterilized tap water were vacuum filtered onto a 4.7 cm Nuclepore membrane (5 µm pores) (Whatman, NJ) placed on a holder; the membrane and holder were placed in the middle of a Nalgene filter funnel with a clamp (Nalgene, NY), and a vacuum flask connected to the funnel was attached to the air pump. The membrane with nematodes was transferred to a petri dish without lid, which was placed on a ceramic holder in an air-tight glass chamber with 72% glycerol solution at the bottom. A MicroRHTemp Data Logger (Mudgetech, NH) was placed on the ceramic holder to collect relative humidity (RH) and temperature data. The chamber was incubated at room temperature for 24 h, which will be referred to as desiccation treatment from here on. Approximately 500 nematodes in sterilized tap water were placed in a small petri dish and incubated at 4 °C for 24 h, and this treatment will be referred to as the cold treatment. Approximately 500 nematodes were transferred in a beaker with 20 ml 500 mM of sucrose solution and were incubated at room temperature for 24 h, and this treatment will be referred to as the osmotic treatment. Five hundred *A. fragariae* in sterilized tap water were incubated at the room temperature for 24 h as a control. After 24 h treatment of the three stress conditions, the nematodes were transferred into sterilized tap water to rehydrate for 24 h. Survival was evaluated by

checking motility with physical stimulation under a stereoscope in 100 individuals arbitrarily selected. All treatments were repeated in the same manner using *Aphelenchus avenae*. The whole experiment was repeated three times.

**DNA extraction and PCR.** Genomic DNA was extracted using Sigma Extract-N-Amp kit (Sigma, St. Louis, MO) from 50 nematodes hand-picked from *A. fragariae* and *A. avenae* under rehydrated condition. The manufacturer's protocol was modified by reducing all volumes to one fourth of the recommended amounts. Fifty nematodes of each species were placed into a 0.2 ml centrifuge tube containing 25 µl of the kit's Extraction Solution. The nematodes were crushed using the tip of a 10 µl pipette tip, followed by adding 7 µl Tissue Prep solution of the same kit to the tube. The tube was vortexed followed by a brief centrifugation to collect contents. The mix was then incubated at 55 °C for 10 minutes followed by incubation at 95 °C for 3 minutes. Next, 25 µl of the Neutralization Solution of the same kit was added to each tube and extracted DNA was used for PCR or stored at -20°C. Reactions of 25µl contained 1x ReadyMix Taq PCR Reaction Mix (Sigma-Aldrich, St. Louis, USA):50 mM of KCl, 1.5 mM of MgCl<sub>2</sub>, 0.2 mM of dNTP, 0.2 uM of each primer, 1.5 units of Taq DNA polymerase and 2 µl of the DNA extracts described above. PCR primers used in this protocol were tps-F: 5'- GAC GAG TAC CCG AAC TGG AA -3', tps-R: 5'- TGC ACA CCA GCATCT CTT TC-3'; lea-F: 5'- CAC TAC CGC TTA CAA CCA ATC -3', lea-R: 5'- ACA GGA ATA TCA CTG ACA GAT -3'; ahn-F: 5'- CAA ATC AAC AAC AAT GCC ACC -3', ahn-R: 5'- AGA GGA TCA TTG CAC GGA ATT -3'; glx-F: 5'- GCG AAC ATG GGA AAA

GTC AAC -3', glx-R: 5' - CAG AGA TGC CTT TGG ACA TTA -3'. PCR cycling parameters were initial denaturation of 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 46 °C for 1 min and 72 °C for 2 mins, the final extension of 72 °C for 5 mins was included at the end. PCR products were visualized on 2% agarose gel stained with ethidium bromide.

***Af-tps* and *Af-glx* sequence analysis.** *Af-glx* and *Af-tps* sequences were obtained from an Illumina RNA-seq project of the foliar nematodes carried out in our laboratory. The transcriptome was obtained from mixed stages of the foliar nematodes under desiccated stress condition and different diets, refer more details of the procedure to Chapter 2. We established a local database with contigs assembled from the Illumina reads and queried the sequence of *Aav-ahn* (accession No. AY340998.1), *Aav-glx* (AY340999.1), *Aav-lea* (EF026241.2) and *Aav-tps-1* (AJ811569.1) against the database with tblastx algorithm. Sequences with a low E value ( $E < 10^{-10}$ ) were extracted from the contigs. Two contigs (contig 778 and contig 20397) homologous to *Ava-glx* were submitted to Genbank with accession No. JN881463 and JN881464 and they were named *Af-glx-1* and *Af-glx-2*, respectively. Three contigs (contig 8670, contig 12103\_1 and contig 12103\_2) homologous to *Aav-tps* were submitted to Genbank with Accession Nos. JN881460, JN881461 and JN881462 and they were named *Af-tps-1*, *Af-tps-2* and *Af-tps-3*, respectively. Open Reading Frames (ORF) for *Af-glx* and *Af-tps* sequences were located using ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>), and predicted protein sequences were analyzed via CLC Protein Workbench 5.7 (CLC bio, Denmark). Protein

sequence alignment of Af-TPS and Af-GLX were ran through ClustalW2 (Larkin *et al.*, 2007) and displayed in Jalview (Waterhouse *et al.*, 2009).

***Phylogenetic analysis of predicted Af-GLX and Af-TPS.*** A phylogenetic tree was constructed from nematode Trehalose Phosphate Synthase sequences by maximum parsimony using MEGA 5 (Tamura *et al.*, 2011). A bootstrap consensus tree was created from 1000 replicates. The proteins used for the phylogenetic analysis and the corresponding GenBank Accession numbers are: Aav-TPS-1 (AJ811568), Aav-TPS-2 (AJ811569), Cre-TPS-1 (EFP05039.1), Cre-TPS-2 (EFP02209.1), Cbr-TPS-1 (XP\_002643682.1), Cbr-TPS-2 (CAP30292.1), Cel-TPS-1 (AJ811573), Cel-TPS-2 (AJ811574), Bm-TPS-1 (EDP32297.1), LI-TPS-1 (XP\_003138904.1), As-TPS-1 (ADY40229.1), and out group Dm-TPS-1 (NP\_608827.1).

***RNA extraction and quantitative reverse transcription PCR (qRT-PCR) of Af-glx-1.***

Gene specific primers of glutaredoxin was designed based on the sequence of *Af-glx-1* using Primer 3 (Rozen and Skaletsky, 2000). The expected size of the product is 183 bp. Approximately 5,000 foliar nematodes were treated under three different stress conditions (desiccated, cold and osmotic) for 24 h as described above. Approximately 5,000 nematodes suspended in sterilized tap water were incubated at the room temperature for 24 h as a control. Total RNA treated with each environmental stress condition without rehydration and control was extracted using RNeasy Mini kit (Qiagen, MD, USA) following instruction of the manufacturer. On-column DNase digestion was performed



during the RNA extraction process with RNase-Free DNase set (Qiagen, MD, USA). Quantitative reverse transcription PCR reactions were set up in 25 µl following the instructions of the QuantiTect SYBR Green RT-PCR kit (Qiagen, MD, USA) on a Stratagene Mx3000P QRT-PCR system, using 0.25 µl of the total RNA from each environmental stress condition or untreated control as templates. Quantitative reverse transcription PCR parameters included initial reverse transcription at 50 °C for 30 min, initial denaturation at 95 °C for 15 mins followed by 40 cycles of 94 °C for 15 s, 50 °C for 30 s, and 72 °C 30 s (at the end of the this step, fluorescence data were collected). The dissociation curve (melting curve) was performed at the end of the program from 60 °C to 90 °C. Partial 28S ribosomal RNA gene (154 bp) was included for all samples as reference for normalization of products. The primers used in the qRT-PCR were glx-F: 5'-ATT AGA TGG CTT GGG CTT CTT G-3', glx-R: 5'-TTG AGA TTG AAG ACC GCA AAG A -3'; and 28S-F: 5'-AGT GGG ACA CTT GGT GTC TGT GA-3', 28S-R: 5'-TCT GAC TTC GTC CTG TTC GGG CA-3'. Four replicates were included for each condition. The standard curve method was used to quantify differences in expression. A dilution series of the "Untreated control" nematode total RNA was used to create standard curves for both the 28S and glutaredoxin genes. Threshold (Ct) values from each sample were converted to the relative quantity based on the standard curve. The expression levels of *Af-glx-1* was normalized by 28S ribosomal gene, and was separately computed in each environmental condition. All amplicons were treated with ExoSAP-IT (Affymetrix, Santa Clara, CA) following the instruction of the manufacturer and sequenced at Clemson University Genomic Institute.

**Statistical analysis.** Survival rate of control was assumed to be 100 %, and all the treatment survival data were normalized for the control for each species. Data of nematode survival between two nematode species were analyzed by one-way ANOVA with JMP 9 (SAS Institute, Cary, NC). Significant differences between species of each environmental stress condition were determined by Student's t test at  $P < 0.05$ .

## **Results**

**Nematode survival under different stresses.** *Aphelenchoides fragariae* and *A. avenae* were observed forming aggregates or 'nematode wool' on the membrane after the desiccation treatments. Nematode body fluid was transparent after the immediate rehydration, and then became darker after a few hours of rehydration. The lag phase was observed after rehydration: 30 min after rehydration, *A. fragariae* started moving and after 2 hours, 90% of the *A. fragariae* were motile. It appeared that 2 h is necessary for *A. avenae* to start moving, and after 4 h of rehydration, 90 % of the *A. avenae* were motile. The survival rate of *A. fragariae* (91.09 %) was significantly higher than for *A. avenae* (77.73 %) under desiccation (Fig. 4.1). The same trend was evident for osmotic stress. No difference in survival rates was found between the two species under cold tolerance, which were both above 90 %.

**Homologs of desiccation related genes.** We were not able to amplify *ahn*, *glx*, *lea* and *tps* genes in the *A. fragariae* genome, while all these genes were amplified in the *A.*

*avenae* genome. However, in the *A. fragariae* transcriptome, 7 cDNA sequences were found homologous ( $E < 10^{-10}$ ) to *Aav-glx* (Table 4.1), 11 cDNA contigs were found homologous to *Aav-tps* (Table 4.2) via local blast. Because some of the sequences were short, we only analyzed the long sequences in this chapter. No cDNAs were found homologous to *Aav-ahn* or *Aav-lea*.

**Characterization of *Af-glx*.** *Af-glx-1* (JN881463,) and *Af-glx-2* (JN881464,) were 372 bp and 764 bp, respectively. Open Reading Frame of *Af-glx-1* is from 47 bp to 371 bp and encodes a protein of 107 aa (Fig. 4.2) with molecular weight of 12.011 kDa and Isoelectric Point (PI) 8.89. Open Reading Frame of *Af-glx-2* is from 76 bp to 744 bp and encodes protein of 232 aa, which has a molecular weight of 26.053 kDa and Isoelectric Point (PI) 9.02. Predicted polypeptide of Af-GLX-1 has Glutaredoxin (GRX) domain from 16 to 96 aa (Fig. 4.2), predicted putative Af-GLX-2 also has GRX domain from 127 to 212 aa and a SelenoproteinS from 4 aa to 94 aa at N terminus, which is missing in putative Af-GLX-1. Putative Af-GLX-1 aligned with Af-GLX-2 from 7 aa to the end, which covered the whole glutathione redoxin (GRX) domain (Fig. 4.3). Though the two putative polypeptide sequences are not identical, they both have RSVP and GGDD conserved sites to bind Glutathione, and Cysteine and Serine as catalytic residues.

**Characterization of *Af-tps*.** *Af-tps-2* and *Af-tps-3* were two splicing isoforms. There were more splicing isoforms in the transcriptome homologous to *Aav-tps*, however, because they were not full length sequences, we are not discussing those short splicing

isoforms in this chapter. The predicted protein Af-TPS-2 is 14 aa longer than Af-TPS-3. Af-TPS-2 and Af-TPS-3 aligned completely (Figure 4.4) except for the 14 aa (sequence SGHSDANGSCSSLW). Af-TPS-1 aligned with Af-TPS-2 and Af-TPS-3 from 302 aa to the C-terminus (Figure 4.4). We only showed the domain structure of Af-TPS-2 (Figure 4.5), with a Glycosyltransferase\_GTB\_type superfamily near N terminus, and a Cof-subfamily domain from 791 aa to 893 aa near C terminus. The predicted molecular weight of Af-TPS-2 is 109.82 kDa, with Isoelectric Point (PI) 5.51.

**Phylogenetic analysis.** In the phylogenetic tree, *Aphelenchoides* glutaredoxin was separated from the genus *Caenorhabditis* (Fig. 4.5). Af-GLX-1 and Af-GLX-2 were not placed in the same clade, which demonstrates the difference between these two putative proteins. The glutaredoxin of *Aphelenchus avenae*, which belongs to the same family as *A. fragariae*, was not close to either of *A. fragariae* glutaredoxin.

The phylogenetic arrangement including trehalose phosphate synthase from different nematodes showed that Af-TPS-1, Af-TPS-2 and Af-TPS-3 form a tight cluster (Figure 4.6), which is close to Aav-TPS-1 and Aav-TPS-2. TPS-1 of the genus *Caenorhabditis* are separated from TPS-2 of the same genus. Bm-TPS-1, Ll-TPS-1 and As-TPS-1 were closely related.

**Differential expression of *Af-glx-1* under stress.** Standard curves of glutaredoxin gene and 28S gene had  $r^2$  of 0.995 and 0.998, respectively. Melting curves of 28S gene and glutaredoxin gene were identical for all the samples. Relative quantities were computed

based on the standard curves (28S,  $y = -3.554 \log x + 3.9$  and  $glx$ ,  $y = -3.322 \log x + 17.47$ ). Expression of glutaredoxin gene under desiccated stress was 2.8 higher than the untreated control (Figure 4.7), and negligible up-regulation (1.3 fold-change) of glutaredoxin was also detected under osmotic stress. Glutaredoxin gene was found down-regulated (0.4 fold-change) under cold stress condition.

## Discussion

Water loss is a challenge for plant parasitic nematodes as they experience periods where they are not in the host tissues and exposed to the dry soil, or as nematodes feed on aerial parts of plants, they risk exposure to air as they climb to infect the foliage or stems of the hosts. However, the ability of plant parasitic nematodes to withstand desiccation varies among the genera and species. Larvae and pre-adults of reniform nematodes *Rotylenchulus reniformis* were unable to survive direct short-term exposure to 97% relative humidity (Womersley and Ching, 1989), whereas, at the same relative humidity, the soil dwelling nematode *A. avenae* can become anhydrobiotic by aggregating (Crowe and Madin, 1975).

*Aphelenchoides fragariae* showed higher survival ability than *A. avenae* under desiccation in our study, but it had a lower survival rate than *D. dipsaci* when compared to the results of a study conducted by Wharton and Aalders (1999). A lag phase was observed for both species, and we measured the survival rate after 24 h rehydration to ensure live nematodes were completely recovered. We did not directly measure the time at which 50% of the nematodes became active after rehydration (T50), however, based on

the observation of motility every 30 min, we predicted the T50 of *A. avenae* is between 2 h and 3 h, whereas the T50 of *A. fragariae* was between 1 h and 2 h. Wharton and Aalders (1999) demonstrated that the length of the lag phase was highly related to the severity of the desiccation and not to the final relative humidity, which suggests that longer desiccation or lower relative humidity might extend the lag phase.

Cold tolerance is considered to be associated with resistance to desiccation stress. Exposure of some nematodes to desiccation before entering sub-zero temperature condition, may remove freezable water from their bodies, and allowing them to survive in anhydrobiotic stages (Wharton, 2011) under sub-zero temperature conditions. Both species showed high survival under the cold stress condition we tested. However, we did not test the nematode survival under both desiccation and cold tolerance, which might decrease the survival rate.

*Aphelenchoides fragariae* has ability to survive under osmotic stress. Based on the analogy with anhydrobiotic plants (Ingram and Bartels, 1996), under osmotic condition, water potential can be maintained at similar level as under desiccation stress, adaptations to desiccations might be involved in osmotic stress resistance. These adaptations include change of primary metabolites and synthesis stress-related proteins such as antioxidants (Ingram and Bartels, 1996).

The up-regulation of *Af-glx-1* under desiccated and osmotic stress conditions in our study indicates oxidative, osmotic and desiccation stresses were imposed. It has been shown that reactive oxygen species (ROS) accumulation is triggered by desiccation (Kranner and Birtić, 2005), and ROS accumulation will lead to oxidative modification of

protein, increasing susceptibility to unfolding, and aggregation (Burnell and Tunnacliffe, 2011). It has been illustrated that survival of bacteria from dry climate soils is highly dependent on the mechanisms that decreases protein oxidation during desiccation (Fredrickson *et al.*, 2008). Glutaredoxin is one of the members of these antioxidant systems reactivating many oxidized proteins by reducing the mixed disulfide bonds formed during oxidative stress. Glutaredoxin genes can be induced in response to various stress conditions like heat and oxidative and osmotic stress in yeast *Saccharomyces cerevisiae* (Grant *et al.*, 2000). *Af-glx-2* encodes a glutaredoxin on the C terminus and Selenoprotein\_S (Sels) at the N terminus, which is a plasma membrane protein and protects the functional integrity of the endoplasmic reticulum against deleterious metabolic stress (Martinez *et al.*, 2008). Not only do organisms across kingdoms have similar strategies to deal with desiccation stress, but also organisms have similar molecular strategies to deal with other different environmental stresses as is the case for glutaredoxin. Similar molecular mechanisms have been described on late embryogenesis abundance (LEA), which is a highly hydrophilic protein with anti-aggregation activity found in *A. avenae* (Browne *et al.*, 2002) and in plants (Ingram and Bartels, 1996).

Trehalose was accumulated in the desiccated nematodes *A. avenae* (Browne *et al.*, 2004; Crowe and Madin, 1975) and it can make up 11% of the nematode dry weight. Trehalose serves as a signaling molecule in some metabolic pathways, and it also protects proteins and cellular membranes from inactivation caused by different stresses, especially desiccation [reviews in (Elbern *et al.*, 2003 )]. There are few pathways for synthesis of trehalose, the best known and most common is transfer of glucose from UDP-glucose to

glucose-6-phosphate to form trehalose-6-phosphate and UDP in the first step with a second step to remove the phosphate and generate trehalose. Trehalose phosphate synthase (TPS) catalyzes the first step in the reaction [review in (Iordachescu and Imai, 2006)]. Eleven contigs were found homologous to *Aav-tps* in the *A. fragariae* transcriptome, suggesting alternative splicing in the mRNA processing.

In conclusion, the foliar nematode *A. fragariae* has the ability to survive under desiccation, which assists explain the overwintering survival ability in dry soil, abscised leaves and dormant buds. Glutaredoxin genes have been identified from the *A. fragariae* transcriptome, and up-regulation of glutaredoxin gene expression has been detected under desiccated and osmotic stresses, suggesting a participation of anti-oxidation mechanisms in resistance to the stress imposed by water loss.



Table 4.1. *Aphelenchoides fragariae* contigs assembled using Trinity program homologous to glutaredoxin gene from *Aphelenchus avenae* Aav-glx.

| <b>Contig No.</b> | <b>Length of contig (bp)</b> | <b>E-value</b>    |
|-------------------|------------------------------|-------------------|
| Contig778_1       | 372                          | 4e <sup>-31</sup> |
| Contig13129       | 322                          | 7e <sup>-26</sup> |
| Contig201         | 455                          | 2e <sup>-22</sup> |
| Contig20397       | 764                          | 6e <sup>-17</sup> |
| Contig6113        | 715                          | 4e <sup>-16</sup> |
| Contig50695       | 317                          | 7e <sup>-15</sup> |
| Contig778_2       | 225                          | 4e <sup>-11</sup> |

Table 4.2. *Aphelenchoides fragariae* contigs assembled using Trinity program

homologous to trehalose phosphate synthase gene from *Aphelenchus avenae* *Aav-tps*.

| <b>Contig No.</b> | <b>Length of contig (bp)</b> | <b>E-value</b>    |
|-------------------|------------------------------|-------------------|
| Contig8670        | 2770                         | 0.0               |
| Contig12013_1     | 3298                         | 0.0               |
| Contig12013_2     | 3256                         | 0.0               |
| Contig12013_3     | 3090                         | 0.0               |
| Contig6143_1      | 1962                         | 0.0               |
| Contig12013_4     | 1191                         | 3e <sup>-54</sup> |
| Contig15020       | 1773                         | 2e <sup>-33</sup> |
| Contig12103_5     | 1024                         | 1e <sup>-29</sup> |
| Contig6143_2      | 258                          | 3e <sup>-28</sup> |
| Contig12103_6     | 291                          | 5e <sup>-23</sup> |
| Contig5667        | 537                          | 1e <sup>-16</sup> |

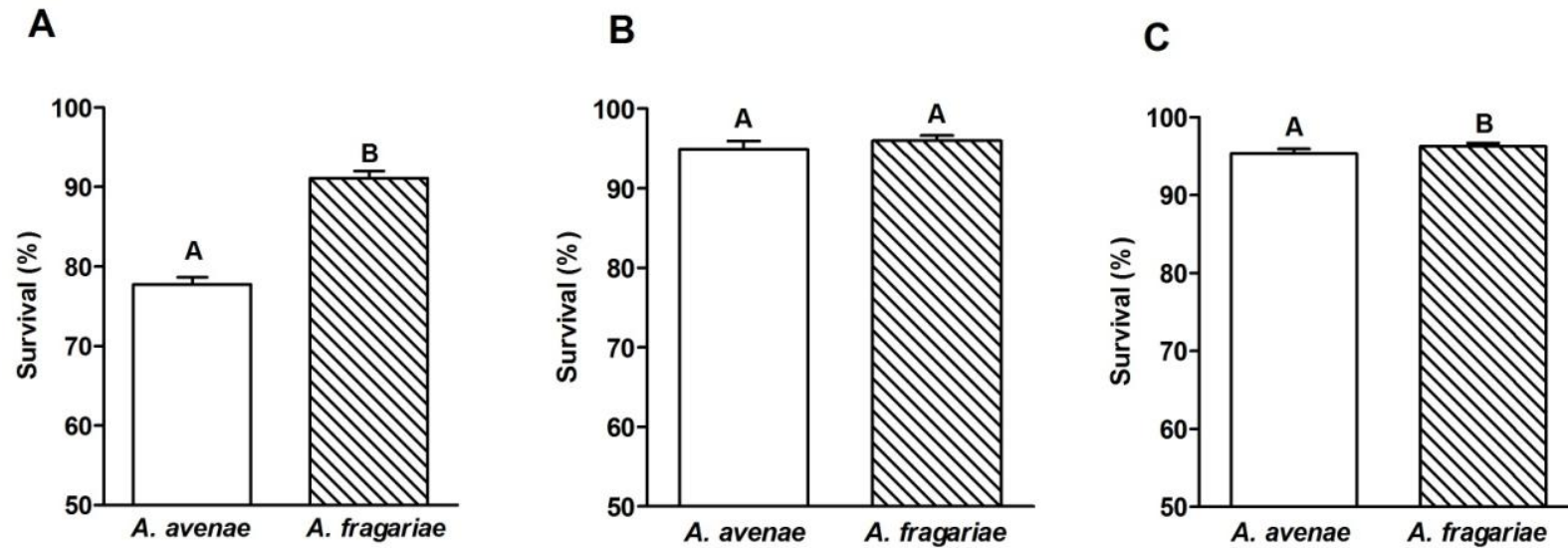


Figure 4.1. Survival of *Aphelenchoides fragariae* and *Aphelenchus avenae* under A. desiccated; B. cold; and C. osmotic stress.

All the survival data were normalized to the untreated control. Different letters indicate significant differences ( $P < 0.05$ )

within the same stress treatment according to the Student's  $t$  test.

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attctctcaaaaagtttcaaagtctatcatcaaacaagagcgaaaaatgtctgttgaaaggctttcgtt
                                     M S V G K A F V

gattcagctatcaagcaatacaaagttggtggttcagcaagtcgtattgtccttactgc
D S A I K Q Y K V V G F S K S Y C P Y C

aagaaagccaaggaagcattggacagttacaattcaaagaaggcgcttttggttgatt      **
K K A K E A L D S Y K F K E G A F G W I

*****
gagattgaagaccgaaagacatggatgaaatccaagactatgtgaaggagttgaccggt
E I E D R K D M D E I Q D Y L K E L T G

gccagatcagtcccaagagtctttattaacaaaaattctttggtggaggtgacgacaca
A R S V P R V F I N Q K F F G G G D D T

aaggctgctcactcaaatggaaaactcgaaaaggcacttcaagaagccaagccatctaatt      *****
K A A H S N G K L E K A L Q E A Q A I #

```

Figure 4. 2. *Aphelenchoides fragariae* glutaredoxin *Af-glx-1* cDNA sequence (GenBank accession JN881463) and predicted polypeptide. Open reading frame is from nucleotide 47 to 371. Glutaredoxin (GRX) domain is boxed (position 16 to 99). Primers designed for quantitative reverse-transcription polymerase chain reaction (qRT-PCR) are marked with asterisks and amplify a fragment of 183 bp. Stop codon is marked with the number sign.

```

Af-GLX-1      -----
Af-GLX-2      1 M I E S I G Q F F E R Y G W Y A L F G A F A F F L I Y Q K F L H D K I Q N L Y E 40

Af-GLX-1      -----
Af-GLX-2      41 N R E L V K Q K K F D E S Q Q Q A N Q E R M E K A R L K M Q E E Y S K Q V Q R E 80

Af-GLX-1      1 -----MS-----VGKAFVD 9
Af-GLX-2      81 L E I K E E Q A Q K R K I Q K L G L S E E L K A N A N K P A R F D G L Q F V K 120

Af-GLX-1      10 S A I K Q Y K V V G F S K S Y C P Y C K K A K E A L D - S Y K F K E G A F G W I 48
Af-GLX-2      121 D Q V A S K P V V I F S K S W C P Y S R K A K Q I L I G S Y R I D S H F Y T V I 160

Af-GLX-1      49 E I E D R K D M D E I Q D Y L K E L T G A R S V P R V F I N Q K F F G G G D D T 88
Af-GLX-2      161 E L D E I E N G E L I Q D A L Q R V T G G R S V P R V F I Q G E F I G G G D D T 200

Af-GLX-1      89 K A A H S N G K L E K A L Q E A Q A I - - - 107
Af-GLX-2      201 E R L H R E N K L S P M L K A A N V K F V E 222

```

Figure 4.3. Sequence alignment of two predicted *Aphelenchoides fragariae*

Glutaredoxin proteins, Af-GLX-1 and Af-GLX-2. Af-GLX-1 aligned to Af-GLX-2 from position 112 to the end. The alignment was performed via algorithm ClustalW2. Boxed amino acids are Glutathione (GSH) binding sites, and arrows indicate catalytic residues of Glutaredoxin.

```

Af-TPS-2/1-961 -----
Af-TPS-3/1-947 -----
Af-TPS-1/1-881 MTRSESQRSMPNSGSSSTSLFTQEHVKQS I QKTLKVVNSE I DENLPLDSY I DQGLE I MVAHWRKRDNTSE I TFQGLS I LEYCLQHSFESTEFFEKFNALGYHTVQFWRAAVPY I FDSDM 120

Af-TPS-2/1-961 -----
Af-TPS-3/1-947 -----
Af-TPS-1/1-881 SHGTKYRDALLFSLTLYDVNTGKNRLKELYAAVPGI RKSLLGVHAKRFGEQYHHLQRRRSASQSLNQSMDSL DNA I SGVK I NASDEDEDSL NKKSKRKMMSMSSEENP I VTDDATSF T S 240

Af-TPS-2/1-961 ----- -MN I DRSKLRSTEV LNMADDNMT I APTTNTLGLPLMRQAMADVMFHVI ADDDVKESKETP 59
Af-TPS-3/1-947 ----- -MN I DRSKLRSTEV LNMADDNMT I APTTNTLGLPLMRQAMADVMFHVI ADDDVKESKETP 59
Af-TPS-1/1-881 GGLANTHFQQRV I NVSNAPPVSLKREKSGEWE I RQSGGGLVSCVDPVMSVYQENMML ANLGMN I DKSCLKSTEV LNLQLEDLST APATNTLGLPLMROPKADVL FHV I ADDDIKETKETA 360

Af-TPS-2/1-961 DQK I VRSGHSDANGSCSSLWQEMSL L GVLN N YNKGNYKLN PVI VQE QDY NVVYGG I SNGLLW PALHNLPEY I ASDYDDHRVLRDHWSSYVRVNYQFA I DSVRNSRPQDF IWI HDYHMLT 179
Af-TPS-3/1-947 DQK I VR ----- QEMSL L GVLN N YNKGNYKLN PVI VQE QDY NVVYGG I SNGLLW PALHNLPEY I ASDYDDHRVLRDHWSSYVRVNYQFA I DSVRNSRPQDF IWI HDYHMLT 165
Af-TPS-1/1-881 EQK I ARSAHSEKLEASSTLWEQMSL L GVLN N YNKGNYKLN PVI VVVEQDY NVVYGG I SNGLLW PALHNLPEY I ASEYDDPRVLRDHWSSYVRVNYQFA I DTVRNSRPQDF IWI HDYHLLT 480

Af-TPS-2/1-961 GMI MQSLDQNL E I GFFLHI PFLPPENFFTKYRI CAFPI LRGLLRFSKVG FQTHRDRAKFLEL I NLHLPTVKI SFDPNLD I HVVTYQGWS CSLGVFPVSI KNEDFLKVAKAAETVKKSHD I 299
Af-TPS-3/1-947 GMI MQSLDQNL E I GFFLHI PFLPPENFFTKYRI CAFPI LRGLLRFSKVG FQTHRDRAKFLEL I NLHLPTVKI SFDPNLD I HVVTYQGWS CSLGVFPVSI KNEDFLKVAKAAETVKKSHD I 285
Af-TPS-1/1-881 GMI MQSLDQNL E I GFFLHI PFLPPDNFFTKYRI CAFPI LRGLLRFSKVG FQTHRDRAKFLEL TNQYLPVTRVTFDSNLD I HIVTYQGWS CSLGVFPVSI KNEDFLKVARAAETVKKSHD I 600

Af-TPS-2/1-961 RQK I LGLKPPDGSRL FFSVERFDYTKGI TEKLNAYKRYFEKYPDRI GKDTLYQVAVTNRSSVDTYRVYQDEC I KLADE I QRLFHSE EYPDWRPL I FETDGLQRSDLVAHYLAMDVG VVTP 419
Af-TPS-3/1-947 RQK I LGLKPPDGSRL FFSVERFDYTKGI TEKLNAYKRYFEKYPDRI GKDTLYQVAVTNRSSVDTYRVYQDEC I KLADE I QRLFHSE EYPDWRPL I FETDGLQRSDLVAHYLAMDVG VVTP 405
Af-TPS-1/1-881 RKK I LDNNSPDGSRL FFSVERFDYTKGI TEKLNAYKRYFEKYPDRI GKDTLYQVAVTNRSSVDTYRVYQDEC I QLAE I QTSFHSDEFPNWKPL I FQTDGLQRTDLVAHYLAMD I GVVTP 720

Af-TPS-2/1-961 RKDGMNLVAKEMMVCNPSAGLV LSTGAGSE I QFTMAGLHPDDGDFNYHRVDDLSDCEHYADTLHAAA I EPEEERARHGQRLHEF I MNND I ERWSQAF LDPGWSHLV I RQSQVETLDDFYT 539
Af-TPS-3/1-947 RKDGMNLVAKEMMVCNPSAGLV LSTGAGSE I QFTMAGLHPDDGDFNYHRVDDLSDCEHYADTLHAAA I EPEEERARHGQRLHEF I MNND I ERWSQAF LDPGWSHLV I RQSQVETLDDFYT 525
Af-TPS-1/1-881 RKDGMNLVAKEMMVCNPMAGLV LSTGAGSE I QFTMAGLHPDDGDFTYHRVENLADSEHYADLVYAAATESEDQRRARHGQRLHEF I MNND I ERWSQAF LDPGWSHLV I RQTTVETLDDFYT 840

Af-TPS-2/1-961 LMMRSRDVRRQ I VERVLKGIPIRPHFGI SLNNAKESLSLACQPGTNTLTLQTSNDPNENTDQQA RFD I KNELDEFKDLTFLKFIQSDDVYNVEEF I NSLQIYHPVSSETFREEVMSAVD 659
Af-TPS-3/1-947 LMMRSRDVRRQ I VERVLKGIPIRPHFGI SLNNAKESLSLACQPGTNTLTLQTSNDPNENTDQQA RFD I KNELDEFKDLTFLKFIQSDDVYNVEEF I NSLQIYHPVSSETFREEVMSAVD 645
Af-TPS-1/1-881 LMMRSRDVRRQ I VERVLKGIPIKNHFSI SLNNAKESLSLAF ----- 881

Af-TPS-2/1-961 LFVDADHFMF FFFDRDGT LKSYSCSY PASVQPAYSGVIQAQFARRCAQTCA I VTTAPMMRI GVL DVST I PEGY YYYGASAGREWF I DPASKFKDQSI PEADLELLDRLFTA I GD LLEEPQ 779
Af-TPS-3/1-947 LFVDADHFMF FFFDRDGT LKSYSCSY PASVQPAYSGVIQAQFARRCAQTCA I VTTAPMMRI GVL DVST I PEGY YYYGASAGREWF I DPASKFKDQSI PEADLELLDRLFTA I GD LLEEPQ 765
Af-TPS-1/1-881 -----

Af-TPS-2/1-961 FRHFTWVGSGLQKHYGHLTVAHQDAFGSVKEHQVKA I DEKIHEI I SNI DPEGSI I EVKETDTDLKI FLKSETGDI FDKGHG I RLLVQHTKCDLANGT I LVCGDSMTDLPMLQACLEANPT 899
Af-TPS-3/1-947 FRHFTWVGSGLQKHYGHLTVAHQDAFGSVKEHQVKA I DEKIHEI I SNI DPEGSI I EVKETDTDLKI FLKSETGDI FDKGHG I RLLVQHTKCDLANGT I LVCGDSMTDLPMLQACLEANPT 885
Af-TPS-1/1-881 -----

Af-TPS-2/1-961 GYYT VVVTTSEDLRQTVRLL CERFNNSNFV FVSCPEVLLGAMAQAT I RE I SICRPRI AQDYE 961
Af-TPS-3/1-947 GYYT VVVTTSEDLRQTVRLL CERFNNSNFV FVSCPEVLLGAMAQAT I RE I SICRPRI AQDYE 947
Af-TPS-1/1-881 -----

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Figure 4.4. Protein sequence alignment of three predicted trehalose phosphate synthase proteins of *Aphelenchoides fragariae* (Af-TPS-1, Af-TPS-2 and Af-TPS-3). The graph was drawn using Jalview.

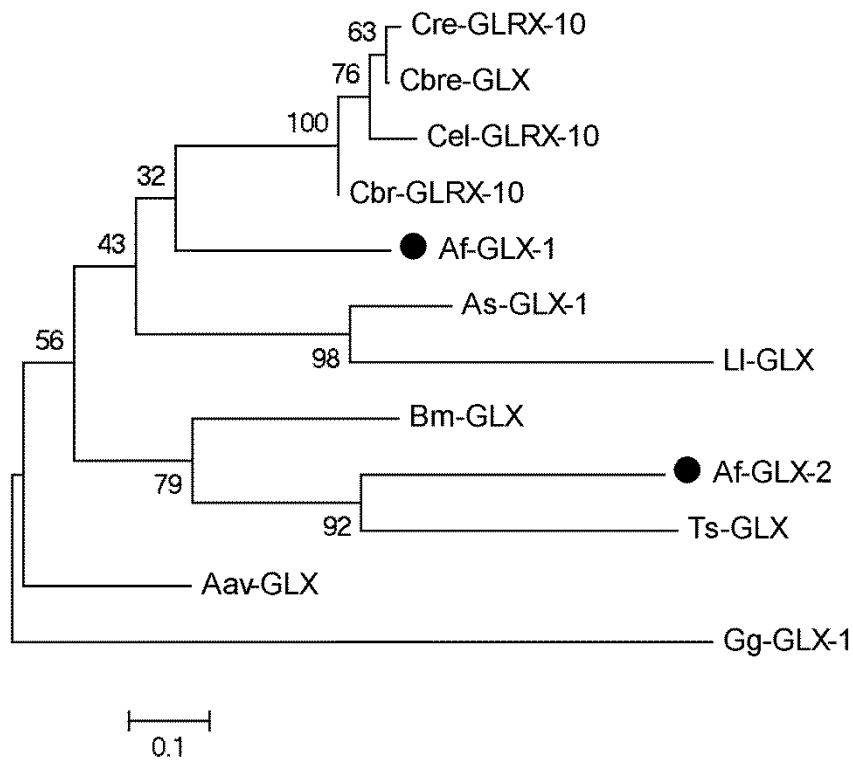


Figure 4.5. Genetic relationships among selected nematode glutaredoxins. The tree was constructed by comparisons of the glutaredoxins listed in the Materials and Methods, using Maximum Likelihood of MEGA 5. The numbers next to the branches indicate the bootstrap value. Abbreviations: Af: *Aphelenchoides fragariae*; Cbr: *Caenorhabditis briggsae*; Cre: *Caenorhabditis remanei*; Cel: *Caenorhabditis elegans*; Aav: *Aphelenchus avenae*; Bm: *Brugia malayi*; As: *Ascaris suum*; Cbre: *Caenorhabditis brenneri*; Ts: *Trichinella spiralis*. Ll: *Loa loa*; Gg: *Gallus gallus*.

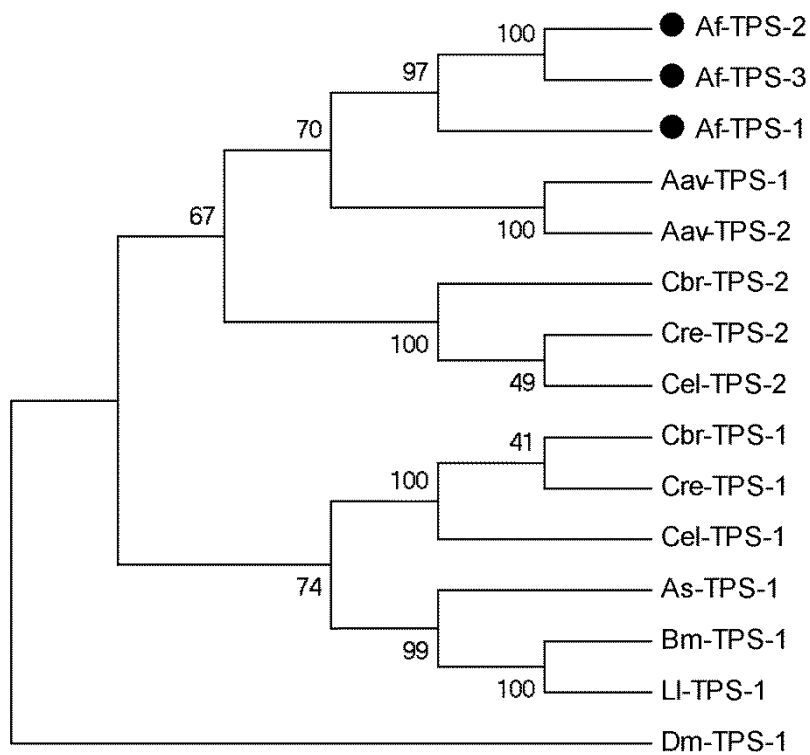


Figure 4.6. Genetic relationship of nematode trehalose phosphate synthases. The tree was constructed by comparisons of trehalose phosphate synthases of nematode species listed in the Materials and Methods using Maximum Parsimony (MP) of MEGA 5. The numbers next to the branches indicate bootstrap values. *Drosophila melanogaster* is the out group. Abbreviations: Af: *Aphelenchoides fragariae*; Aav: *Aphelenchus avenae*; Cbr: *Caenorhabditis briggsae*; Cel: *Caenorhabditis elegans*; Cre: *Caenorhabditis remanei*; As: *Ascaris suum*; Bm: *Brugia malayi*; Ll: *Loa loa*; Dm: *Drosophila melanogaster*



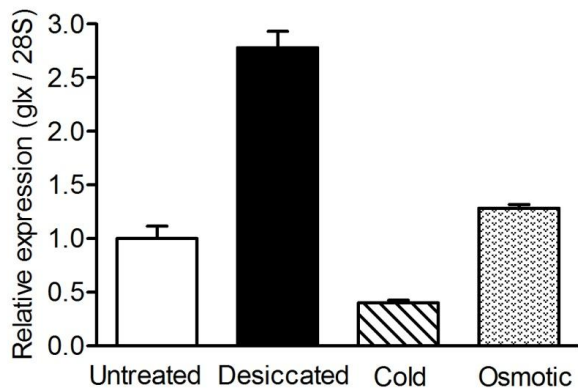


Figure 4.7. Differential expression of glutaredoxin *Af-glx-1* of *Aphelenchoides fragariae* under three stress conditions. The differential gene expression was detected by quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Expression levels of *Af-glx1* were normalized by 28S ribosomal gene. The relative expression level was separately computed for each stress condition. Four replicates were included for each condition. Error bars indicate the standard deviation.

## CONCLUSION AND FUTURE RESEARCH

Standardization of a protocol to evaluate the resistance of hosta cultivars to foliar nematode is a key step of breeding hostas for resistance against this pest. In order to develop a protocol, the effect of inoculum type (foliar nematodes maintained on fungus vs. maintained on plants), inoculation method (with injury vs. without injury), and harvesting methods were tested. I estimated the correlations between nematode reproduction and symptom severity. The variability of the correlation between nematode reproduction and symptoms on hosta leaves highlighted the importance to measure both parameters. More importantly, I demonstrated that there is a loss of virulence of foliar nematodes maintained and increased on fungus cultures, which is related to down expression of  $\beta$ -1,4-endoglucanase (*Afr-eng-1*).

When individual nematodes from hosta plants were transferred to a fungus culture (diet-change), expression of the *Afr-eng-1* decreased 1,812- fold after five generations on the fungus diet. Diet was associated with changes in foliar nematode body size. After five generations on fungus diet, foliar nematode body size became shorter and wider compared to the parents. Additionally, fewer individuals in the diet-changed nematodes were detected bearing *Afr-eng-1* compared to the nematodes always on plant diet, which suggests potential selection acting on the diet-change process.

Illumina sequencing technology was used to study molecular mechanisms of two attributes which are essential for foliar nematodes of being successful plant pathogens: 1.

facultative feeding, (e.g. ability to feed on fungi or plants) and 2. desiccation tolerance in overwintering survival. Illumina sequencing generated 43,364,338 reads of 100 bp length. *De novo* assembly with Trinity program produced 50,686 unigenes, of which 20,558 unigenes were annotated using Blast2GO program. A large portion of *A. fragariae* unigenes were found homologous to genes of well-studied nematode species: *Caenorhabditis* spp. and *Ascaris sum.* I used RSEM and DESeq programs to analyze genes differentially expressed under diet-changed from plants to fungi and under desiccation stress conditions. A number of genes associated with redox function of the cells were abundantly represented under the desiccation condition compared to the hydrated control, like butanediol dehydrogenase, aldo-keto reductase, and glutaredoxin. Meanwhile, trehalose phosphate synthase was found to be up-regulated under desiccation stress, which suggests a role of the disaccharide as a protectant of membrane integrity under water loss stress. Chaperonin containing tcp 1 delta subunit was also over-expressed induced by desiccation, which implies that the anti-aggregation role of chaperons could be facilitative in foliar nematode tolerance to desiccation. Expression of some carbohydrate metabolic genes were found highly decreased after 5 generations on a fungal diet, e.g beta-endoglucanase and glucosamine-fructose-6-phosphate aminotransferase, which indicates different metabolic pathways may be operating with different dietary demands of *A. fragariae*. Illumina sequencing provided a comprehensive and valuable resource to study gene expression, RNA splicing and metabolic pathways of foliar nematodes, which will assist further research of this nematode and development of new management strategies.

Based on the contigs assembled and genes differentially expressed under desiccation and diet-change, I was able to characterize important genes associated with desiccation and facultative feeding attributes of the foliar nematodes. The full length sequence of *Afr-eng-1* was found to encode a glycosyl hydrolase family 5 (GHF5) protein, and quantitative reverse transcription PCR (qRT-PCR) was used to verify that this gene was differentially expressed. The same technique was used to study the differential expression of a glutaredoxin (*Af-glx-1*), which was found to be up-regulated under desiccation and osmotic stress, which suggests that anti-oxidative activity is involved in osmotic and desiccation stresses.

Further study and verification of expression of genes by and diet change with an independent methodology of Illumina sequencing are necessary to create a more complete understanding of facultative feeding and desiccation attributes. It will be important to compare the foliar nematode transcriptome to the transcriptome of other plant- parasitic and fungivore nematodes. The comparison will provide more evolutionary clues to the acquisition of plant parasitism of nematodes. Further population genetic analysis is needed to clearly conclude whether the reduced expression of *Afr-eng-1* in the diet-changed foliar nematodes is due to population selection or individual gene expression changes. I only had success with changing the diet of foliar nematodes from plant to fungus and maintaining good proliferation on fungus diet. It will be interesting to investigate the reverse, or changing the diet from fungi to plants and observing changes in nematodes back to the original diet.

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