ABSTRACT

HEIKEN, JULIA ANNE. The Effects of Fluopyram on Nematodes. (Under the direction of Charles Opperman).

Nematodes are microscopic roundworms that can live freely in many different environments or as parasites of animal or plants hosts. Plant-parasitic nematodes are significant pests, resulting in an estimated US$100 billion annual global crop losses. This high level of impact on agriculture is due to many reasons including the lack of available control methods such as registered nematicides. Historically successful chemical nematicides have lost registration due to environmental and human health risks. As a result, growers often are faced with a need for safe but effective chemical control method for plant-parasitic nematodes but few available options. Fluopyram (Velum®), a fungicide, was discovered to have nematicidal properties. The mode of action for fluopyram against fungi has been determined to be a succinate dehydrogenase inhibitor (SDHI), however the mode of action in nematodes has yet to be confirmed. The first objective of this work was to confirm the mode of action in nematodes by using a succinate dehydrogenase (sdh1) mutant of Caenorhabditis elegans. Nematodes incubated in fluopyram solution appeared dead (straightened and non-motile) and the EC₅₀ for wild type C. elegans was found to be 11.4 ppm while the EC₅₀ for knockdown the sdh1 mutant was more sensitive at 4.31 ppm fluopyram. The oversensitive response observed in the sdh1 mutant compared to wild type C. elegans confirmed that succinate dehydrogenase is the likely target for fluopyram mode of action in nematodes. The second objective was to assess the potential ovicidal effect of fluopyram for C. elegans as well as the plant-parasitic nematodes species Meloidogyne incognita and Heterodera glycines. It was determined that there was significant (p<0.05) effect on hatch rate at 25, 35, and 50 ppm fluopyram for M. incognita and H. glycines but no significance in C. elegans.
hatch rate, due to the reduced permeability of eggshells of *C. elegans* to fluopyram. The third objective was to determine potential reversible effects of fluopyram on *C. elegans* and *M. incognita*. *C. elegans* exposed to fluopyram for 24 hours did not recover while *M. incognita* exposed to fluopyram for 1 hour did recover, suggesting that time of exposure to fluopyram was critical to durable activity. The final objective was to assess the potential affect soil type might have on fluopyram’s ability to control nematode populations in plants inoculated with *M. incognita* or *H. glycines* eggs and grown in three different soil types. The ability of fluopyram to reduce plant root infection by *M. incognita* or *H. glycines* was not significantly reduced in soil types ranging from coarse sand to the high organic matter potting mix.
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The Effects of Fluopyram on Nematodes

by

Julia Anne Heiken

A thesis submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the degree of Master of Science in Plant Pathology

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DEDICATION

I would like to dedicate this document and all the work that went into it to my parents Jeff and Sondra Heiken. Thank you for support through my life and believing in me.
BIOGRAPHY

Julia Heiken was born on April 28, 1993 in Orlando, Florida. She completed her Bachelors of Science degree in Plant and Soil Science with a concentration in Crop Biotechnology at North Carolina State University in 2015. In the fall of 2015, Julia accepted research program at North Carolina State University in the Department of Entomology and Plant Pathology to pursue her Master of Science. She was awarded the Bayer CropScience Fellowship in Plant Pathology to research the effects of fluopyram on nematodes.
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I would like to thank my family and friends for their undying support during my graduate career and throughout my life leading to this opportunity as well. Their emotional support has been essential in my success as a graduate student. I would like to thank my dog, Jack, for keeping me sane and happy. I am especially thankful to my committee members, Drs. Charles H. Opperman, Eric L. Davis, and Michael R. Schwarz. Their guidance during this project has been essential for my success and they have also given me a great deal of advice and support.

Outside my committee, I have been mentored by Chunying (Lisa) Li and Dave Dickey during graduate career that I would like to thank. Lisa, who has served as lab manager, has supported me throughout my entire time at NC State with her knowledge of plants, nematodes and more in addition to being a great emotional support. Dave Dickey has served as my CALS statistical consulting while working on my graduate degree. I would like to thank the undergraduate assistants who helped me including Andrew Fiest, Chelsea Senter, Joshua Berning, and Cole Dunbar.

Finally, I would like to thank Bayer CropScience for selecting me as the Bayer CropScience Fellow in Plant Pathology. This has provided me with financial support as well as additional opportunities for professional development.
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LITERATURE REVIEW

What is a nematode?

Nematodes are roundworms belonging to the phylum Nematoda and include about 30,000 described species with estimations of the total number of species to have ever lived to be a million or more (Kiontke and Fitch, 2013). The name comes from the Greek word for thread ‘nema’ as nematodes have a threadlike body (Kiontke and Fitch, 2013). Nematodes are not segmented but instead have the body shape of a tube within a tube (Kiontke and Fitch, 2013). Among these species there are many nematodes with different life styles, diets, and habitats that can be characteristic of a nematode species. The majority of Nematoda are free-living nematodes which includes the model organism Caenorhabditis elegans. These free-living species feed primarily on bacteria and can be found in a variety of habitats that range from soil to freshwater. In contrast to the free-living nematodes, there are parasitic nematodes which can be found using plants, insects, and animals as hosts (Perry and Moens, 2011). All nematodes embryonate within an eggshell and must complete four juvenile (aka larval) stages to reach reproductive maturity, with molting of their outer cuticle between each stage (Kionthke and Fitch, 2013).

Caenorhabditis elegans as a model organism.

Caenorhabditis elegans, a free-living nematode, is a representative of this large order and used as a model organism for studying nematodes, animals, multicellular biology, genetics, and genomics (Felix and Braendle, 2010). The first identification of C. elegans occurred in 1897 in soil with rich humus and was as a result considered to be a soil nematode.
(Felix and Braendle, 2010). It has been demonstrated to prefer microbe-rich habitats such as decaying plant matter (Felix and Braendle, 2010). The development of *C. elegans* as a model organism is due to the work of Sydney Brenner starting in the 1960’s and his development of the understanding of the organism’s genetics (Corsi et al. 2015). The benefits of working with *C. elegans*, including its small size, transparency, quick life cycle, ability to easily develop large population size, and ease in genetic manipulation, is what led Brenner to develop *C. elegans* as a model organism and what drives many scientists to continue to work with the species. The large body of work investigating the genetics, molecular mechanisms, behavior and more has persisted as a result of development of it as a model organism (Corsi et al. 2015).

The expansion of this body of work has been organized through many databases dedicated solely to the study of *C. elegans* such as wormbook.org and wormbase.org. In 1998, The *C. elegans* Sequencing Consortium completed the sequencing of the *C. elegans* genome (*C. elegans* sequencing Consortium, 1998). The completion of this project allowed for the analysis of all genes present in the organism and was the first multicellular organism to have its genome sequenced (*C. elegans* sequencing Consortium, 1998). *C. elegans* makes a particularly good model organism for studying the effects of nematicides as molecules that affect *C. elegans* are likely to similarly affect parasitic nematodes. This notion was investigated by Burns et al. (2015) and it was found when screening compounds against nematodes, some compounds are lethal to both *C. elegans* and parasitic nematodes while being inactive against vertebrates, an important component of safe nematicides. In addition to
similarities in their sensitivities, *C. elegans* can be a preferred precursor in the study of plant-parasitic nematodes as many obligate biotrophs are difficult and time consuming to culture, especially compared to *C. elegans*.

**The life cycle of *Caenorhabditis elegans***.

In the life cycle of *Caenorhabditis elegans*, a single hermaphrodite produces both sperm and oocytes promoting either self-fertilization or outcrossing with males. From this the fertilized eggs, each a 30 x 50-μm oval shape, remain and develop internally before being laid. A fertilized egg develops into the first larval stage (L1) within the hermaphrodite body, the stage from which they hatch from the egg. The cycle continues with molts of the cuticle and development into the remaining larval stages (L2-4) (Lewis and Fleming, 1995).

Due to the *C. elegans* hermaphroditic reproductive scheme there is variations in sex ratios within populations. The progeny of a self-fertilized XX hermaphrodite will be almost exclusively hermaphrodite progeny with rare XO males. There are typically 280 offspring from the hermaphroditic reproduction. In the occurrence of fertilization by males, the resulting progeny are both male and hermaphrodites with about 1000 offspring by sexual reproduction.

As the life cycle continues there is an alternative stage that can take place of the third larval stage known as a dauer. Dauer is a survival stage in which the nematode has a sealed mouth to stop feeding as well as a tough cuticle and can survive for several months. Dauers can return to the developmental process when reintroduced to food and will molt into a fourth stage larva. Dauers are about 400 μm long while adult *C. elegans* hermaphrodites are
about 1 mm long (Lewis and Fleming, 1995). The life cycle duration is affected by temperature, food source, and environment and optimally takes 90 hours to go from the moment an egg is laid to that nematode laying an egg of their own at 15°C while the cycle will take 45 hours at 25°C (Porta-de-la-Riva et al., 2012).

With the discovery of two additional layers, to the originally investigated three layers, of the \textit{C. elegans} eggshell, it is thought that there are five total layers of the eggshell (Olson et al., 2012). There has been extensive study of these layers to help understand the influence each one has on embryo development. The outermost is the vitelline layer (VL) and can be removed by sodium hypochlorite. The inner most layer is considered to be the osmotic/permeability barrier for the \textit{C. elegans} embryo. The eggshell in \textit{C. elegans} is the extracellular coat protecting the embryo and impermeable to small molecules (Olson et al., 2012)

Many natural and induced mutants of \textit{C. elegans} exist that have utility in genetic and functional assays. Means in which mutagenesis can be induced include chemical, radiation, genetic screen as well as reverse genetics techniques. This allows a look at specific aspects of the organism, such as determining the mode of action of a nematicide (Kutscher and Shaham, 2014). The Caenorhabditis Genetics Center (CGC), supported by the National Institutes of Health – Office of Research Infrastructure Programs, is a resource in which \textit{C. elegans} can be acquired. Wild-type and mutant strains of \textit{C. elegans} are maintained by the CGC.
Plant-parasitic nematodes

Plant-parasitic nematodes are obligate biotrophs that survive off the nutrients of plants as either all or part of their food source. There are over 4,100 species of plant-parasitic nematodes described (Jones et al., 2013). Plant-parasitic nematodes have four developmental stages separated by molts, like all nematodes, and the first molt always occurs within the egg and the second-stage juvenile (J2) hatches from the egg (Agrios, 2005). The majority of plant parasitic nematodes feed from plant roots in soil, but some species can feed from aboveground plant tissues. Plant-parasitic nematodes can be grouped into ectoparasites and endoparasites. Ectoparasitic nematodes remain outside the plants and can cause damage to the roots of the plant by feeding externally. Endoparasites, which can be sedentary or migratory, enter into the roots and cause damage through movement and feeding (Williamson and Hussey, 1996). The majority of plant-parasitic nematode species belong to the suborder Tylenchina, including the southern root-knot nematode, *Meloidogyne incognita*, and soybean cyst nematode, *Heterodera glycines*.

*Meloidogyne incognita* and *H. glycines* are sedentary endoparasites that enter and feed on the host from within from a specific feeding site and do not move throughout the host. Opposite to sedentary are migratory endoparasites which move throughout the roots for their complete life cycle, causing physical damage (Agrios, 2005). *M. incognita* and *H. glycines* are obligate biotrophic parasites that survive off a live host and require parasitism of a host to complete their life cycles (Jones and Goto, 2011). While the majority of *M. incognita* and *H. glycines* life stages are spent within the host, the hatched infective J2 stage
is motile allowing for dispersal of the nematodes in soil and other environments (Williamson and Hussey, 1996). All plant-parasitic nematodes have a protrusible oral stylet, a hollow needle-like structure, that is used to ingest nutrients from the plant as well as to secrete effector proteins that are essential to the infection process (Lambert and Bekal, 2002, Mitkowski and Abawi, 2003). Nematode feeding activity, mechanical damage, and predisposition to secondary plant pathogens all contribute to the adverse affects on host plant growth and potential crop yield.

Plant-parasitic nematodes can reproduce in various ways depending upon species -- amphimixis, facultative meiotic parthenogenesis, and obligate mitotic parthenogenesis. Amphimixis is meiosis which occurs after sperm from male fertilizes female oocytes. Facultative meiotic parthenogenesis is reproduction when amphimixis occurs when males are present but in their absence parthenogenesis occurs in which meiosis occurs within oocytes. Obligate mitotic parthenogenesis is parthenogenic reproduction in which males are not involved and one nuclei in the oocyte deteriorates while the other remains intact to become the embryo. The major species of *Meloidogyne* spp. reproduce through either form of parthenogenesis (Chitwood and Perry, 2009). In contrast, reproduction by *H. glycines* and most cyst nematode species is almost exclusively by amphimixis.

**Significance of Plant-Parasitic Nematodes**

Plant-parasitic nematodes cause an estimated $US100 billion in annual global agricultural damage, but this is considered to be an underestimate (Jones et al., 2013). This economic value equates to an approximate 8-15% crop loss due to nematodes (Kionthke and
Fitch, 2014). An inflation-adjusted estimate of $10 billion in the United States and $125 billion globally in crop losses due to nematodes was reported in 2003 based on the 1987 report of $77 billion in crop losses globally (Chitwood, 2003; Sasser and Freckman, 1987). Some species of plant-parasitic nematodes result in indirect cost due to quarantines, cost of management, and predisposition to secondary plant diseases. Through quarantines, certain nematode species are prohibited by many countries putting limitations on import/export markets for farm products (Moens et al., 2009).

There are few comprehensive assessments of yield losses caused by nematodes but there are various small scale assessments looking primarily at the level of nematode damage within a region and/or on one crop of interest. The Koenning et al. (1999) published summary of crop losses in 1994 due to nematodes was created including information on nematode losses in 35 states in field corn, soybean, wheat, rice, grain sorghum, sugarcane, cotton, peanut, tobacco, vegetables, fruits and nuts, and golf greens. Numerous nematode species were included, and when available, the control methods used in the state were included as well. Tobacco and cotton production, for example, has been highly reliant on nematicides (Koenning et al., 1999). In the Jones et al. (2013) review of the ten most important global plant-parasitic nematode genera, they ranked *Meloidogyne* and cyst nematodes, including *Heterodra* and *Globodera*, as the top one and two most important nematode taxa, respectively. In determining the importance of the nematodes, Jones et al. (2013) considered the economic damage these nematode species are responsible for as well as the contribution the research of these nematodes has made toward understanding
molecular plant nematology (2013). While the ranking of plant parasitic nematodes has been more recent, it is less comprehensive than that of Koenning et al. (1999) and this is largely due to the time and resources required to compile such extensive information. However, this does leave a need for more recent compilations like Koenning et al. (1999), they are a useful tool in presentation of work and proposal writing.

**The Life Cycle of *Meloidogyne incognita***

Second-stage juveniles (J2) of *M. incognita* hatch from eggs in soil and use chemotactic signals to locate and migrate towards host roots (Yang et al., 2016). The J2 penetrate plant roots completely near the zone of elongation and migrate intercellularly to reach the root vascular cylinder. The J2 identify root pro-vascular cells of the root and secrete effectors from their stylet to induce the transformation of plant cells into feeding sites called giant-cells (Jones and Goto, 2011). Giant-cell formation occurs by the induction of nuclear division without cytokinesis resulting in several enlarged multinucleated cells surrounding the head of the nematode (Haegeman et al., 2012).

Upon development of the giant cells, the root-knot nematode can begin to feed, swell and become sedentary, using the photosynthates that accumulate in the giant cells to continue its development (Moens et al., 2009). The nematode undergoes three molts before developing into an adult. During the J3 and J4 stages, the nematode does not feed and moves through these stages quickly (Karssen et al., 2013). The developing adult female swells into a pyriform shape while feeding within the root and at reproductive maturity it lays 500-1,000 eggs in a gelatinous matrix on the surface of the root (Jones and Goto, 2011). Males revert to
vermiform shape, cease feeding and exit the root approximately three weeks after infection (Liu et al., 2007), and only fertilize females in sexually-reproducing species. The *M. incognita* life cycle is 25 days at 27°C (Agrios, 2005) and multiple generations can occur within most crop growing seasons.

The primary and visible symptom of plant root infection by *Meloidogyne* spp. is formation of intercalary galls (knots) surrounding the feeding sites that nematodes induce within host roots. Gall formation occurs as a result of root tissues undergoing hyperplasia around, and hypertrophy within, giant-cells. Plant growth regulators including auxins and cytokinins have been linked to development of giant cells and gall formation in roots (Karssen et al., 2013). Root-knot gall formation varies significantly from plant to plant and can influence the size of the root system but typically occur one to two days after a J2 penetrates the root (Karssen et al., 2013). Necrosis and rotting of roots additionally becomes common in *Meloidogyne* spp. infections toward the end of the growing season. In tuber or tuber-like structures underground, distortion and cracking can occur with small swellings on the surface of the structures (Agrios, 2005). A listing of plants that were susceptible to *Meloidogyne* spp. was compiled by Wesemael et al. (2010) and more plants were susceptible to *M. incognita* than any other species in the genus. *Meloidogyne* spp. infection on non-**Mi** resistant tomato, often referred to as the universal host of root-knot nematodes, can result in yield loss between 25 to 100% (Seid et al. 2015). *Meloidogyne* spp. are often found in clusters known as foci in fields due to the nematode’s inability to move quickly through a field (Mitkowski and Abawi, 2003). Above ground plant symptoms of infection by root-knot
nematodes include stunting, incipient wilt, chlorosis and necrosis, and are often difficult to distinguish from other factors that include similar symptoms. The level at which nematodes specifically affect crop production is difficult to assess in developing regions of the world (Nicol et al., 2011).

**The Life Cycle of *Heterodera glycines***

The life cycle of *H. glycines* begins as an egg in the soil that will then hatch as a second-stage juvenile. Like all plant-parasitic nematodes, the first molt of the nematode’s life occurs while still in the egg (Niblack, 2005). The infective second-stage juvenile then searches for a host root presumably through chemolocation (Yang et al., 2016). Once a soybean root is located the nematode produces enzymes which it will secrete to allow it to penetrate a soybean plant root and migrate intracellularly to the root vascular cylinder (Davis et al., 2000). Effectors from the stylet of the nematode are secreted into a chosen root vascular cell to form an initial syncytial cell. Additional surrounding cells are recruited to join the initial cell into forming a syncytium as the cyst nematode feeding site (Niblack, 2005). The recruited cells are fused to the initial cell and the nematode produces effectors to degrade the cell walls, unlike giant cells that are produced through abnormal cell division (Davis et al., 2000). The syncytium is maintained as a metabolic sink for the nematodes to feed off while they undergo sedentary development to the swollen female and vermiform male life stages (Niblack, 2005).

The later molting stages take place at varying rates depending on the sex of the nematode with females taking longer. During these stages the nematode loses the vermiform
shape and swells. Upon reaching the adult stage, males return to a vermiform shape and exit the root. Adult stage females continue to swell into a lemon shape and to the point that they will partially protrude outside of the root. The females will produce a gelatinous matrix in which some of the eggs the nematode has produced will be deposited. The majority of eggs produced will stay within the female’s body which eventually dies (Niblack, 2005). The females body wall darkens from yellow to dark brown and hardens to become the protective “cyst” which gives the genus its common name (Williamson and Hussey, 1996). This cyst is a survival structure for the eggs. The life cycle from infective second-stage juvenile to viable egg takes 21 to 22 days under ideal conditions (Niblack, 2005).

Symptoms of *H. glycines* include chlorosis, leaf drop, decreased flowering, stunting, wilting, decreased root system size and decreased bacterial nodules on roots (Agrios, 2005). Damage of the plant caused by plant-parasitic nematodes can increase the plant’s susceptibility towards secondary infections (Moens et al., 2009). *H. glycines* can reduce yield up to 30% without causing symptoms that are obvious (Niblack, 2005).

**Management of Nematodes**

As *H. glycines* cannot be eradicated from the soil, the key to control is prevention of spread of the nematode to uninfested regions. Compromises to this practice can occur due to ease of dispersal of the cysts which can be moved by movement of soil, roots, flood water runoff, on machinery or even through wind. Reduction of these spreads can be achieved through general agricultural sanitation practices (Turner and Subbotin, 2013). Use of nematode-free planting material is another way to avoid introduction of nematodes into a
system in which they are not present (Viaene et al., 2013). The creation of phytosanitary programs have increased control of plant parasitic nematodes but successful programs require expertise on identification of the nematode which can often be present and easily undetected. A well-rounded understanding of the biology of the nematode is also crucial in developing these programs (Hockland et al., 2013). *M. incognita* is widespread and as a result is not commonly regulated but other *Meloidogyne* species are regulated. Alternatively, *H. glycines* was regulated by 55 countries in 2011 including Canada, Argentina as well as others (Hockland et al., 2013).

Cultural practices used to in crop systems to manage plant-parasitic nematodes include crop rotation, fallow soil, soil solarization, soil steaming, soil heating, soil flooding and additions of soil amendments (Agrios, 2005; Viaene et al., 2013). The use of non-host crops can sometimes be used if a specific species is present in a field that is not susceptible to all plants. However, this alternative is often not economically viable for farmers in regions where a specific crop is preferred or if specific equipment purchases are too expensive (Mitkowski and Abawi, 2003). Cover crops in field production such as marigold and Poaceae have been investigated for *Meloidogyne* spp. control. The inclusion of a cover crop can have benefits to the soil quality as well but are not always a viable economic choice for growers (Mitkowski and Abawi, 2003; Djigal et al. 2012). Legume species have also been used as cover crop or in rotation to reduce nematode populations as well as incorporation of dried tissue of legumes as a green manure to reduce nematode populations (Morris and Walker, 2002). Oka (2009) reviewed the use of organic soil amendments to decrease nematode
population and includes assessment of plant residues, animal manure, chitinious materials, compost as well as others. In some circumstances the timing of planting can be manipulated to avoid the nematode’s peak season. For example, carrots grown in California have adapted a later planting date to avoid active populations of *M. incognita*. This practice can be beneficial to other pests and pathogens as it changes the crops exposure time (Viaene et al., 2013). Maintenance of a healthy crop by incorporation of proper nutrients and moisture has been shown to minimize soybean damage from nematodes and increase yield (Niblack, 2005).

The degree of success in crop rotation is based largely on the host range of the nematode and the length of rotations necessary for control (Viaene et al., 2013). Crop rotation can be a viable source of management for *H. glycines* due to its relatively narrow host range, however with *M. incognita* this is not the case as it has a wide host range (Turner and Subbotin, 2013). For *H. glycines*, rotation with non-host crops, such as corn, as well as rotation of resistant soybean cultivars between soybean plantings is essential. Of the resistant soybean cultivars rotated, the grower should consider rotating cultivars that received their resistance from different genes to truly maximize the effectiveness of rotations (Niblack, 2005). Incorporation of soybean cultivars containing different forms of resistance can decrease the time between rotations necessary for effective control. While this can be an effective method of control it is not commonly practiced due to decreased yield in resistant varieties (Viaene et al., 2013). Suggested durations of rotations have changed over the years with recommendations ranging from 1 to 2 years to a 5 year minimum (Dropkin, 1984;
Niblack, 2005). Use of a 1-year rotation has been reported to result in a 75% reduction of *H. glycines* population and a 2-year rotation has been reported to result in a 92% reduction of *H. glycines* population (Dropkin, 1984). Various studies of crop rotation of soybean have shown that one year rotations do not change population densities which has lead to the longer rotation recommendations (Miller et al., 2006; Porter et al., 2001; Chen et al., 2001). Similar to the use of certain plants as cover crops, rotation of plants such as *Brassica* spp. can reduce nematode population as their root exudates and chemicals released when decomposing are nematicidal (Miller et al., 2006). In many cases economics are the driving force in the rotations practices a farmer will employ (Niblack, 2005). In some cases, use of rotation is compromised when expensive equipment or subsistence farming limits options (Viaene et al., 2013).

Development of resistant host plants has been attempted for many pathogens and nematodes are no exception. A resistant cultivar is successful if it results in a restriction of reproduction of the nematode to little or none. Tolerant cultivars have the ability to withstand or recover from damage caused by nematode (Williamson and Roberts, 2009). A resistant cultivar is ideal, as well as accepted by farmers, when it has similar agronomic qualities to susceptible cultivars, such as yield potential, without being susceptible to the nematode (Starr et al., 2013). Resistance can arise from either preformed or induced resistance; preformed resistance exists when an uninfected plant has a characteristic expressed at all times that will result in resistance while induced resistance occurs after invasion of nematode into the plant. Mechanisms in which resistance can occur include phytoalexin accumulation, cell wall
fortification, hypersensitive reaction, and accumulation of pathogenesis-related proteins (Huang, 1998). Induced resistance occurs when a gene present in the host plant has a response to the nematode infection that blocks or suppresses a critical step in the infection process. The best-characterized nematode resistance gene in tomato is the \textit{Mi-1} gene that confers resistance against \textit{M. incognita}, \textit{M. javonica}, and \textit{M. arenaria}. \textit{Mi-1} has been incorporated into tomato cultivars that are commonly grown but efficacy of its resistance has limitations at high soil temperatures (Williamson and Roberts, 2009). Crops with cultivars resistant to \textit{M. incognita} include apricot, common bean, cotton, cowpea, peach, tobacco, tomato and walnut (Starr et al., 2013). Crops have been bred for \textit{H. glycines} resistance since its discovery and has resulted in various developments of cultivars resistant to field populations. Peking was one of the original cultivars commonly selected for use in breeding for resistance to \textit{H. glycines} due to its additional agronomic characteristics (Anand et al., 1998). Over time nematode populations have been able to overcome resistance to cultivars and it is recommended to use rotation among resistant cultivars with a focus on rotating among cultivars that derived their resistance from different sources (Niblack, 2005).

Genetically modified host resistance can be derived by broadening \textit{R}-gene resistance, targets in the nematode plant-interaction, targets to the nematode directly and targets to the nematode feeding site (Cottage and Urwin, 2013). For engineered resistance that would require ingestion of active ingredients by nematodes, a feeding tube at the opening of the stylet is considered to function as a molecular sieve with size exclusion limits. Crystal (cry) proteins produced by of \textit{Bacillus thuringiensis (Bt)} have been used as biopesticides as they
are well known for their insecticidal properties (Atkinson et al., 2003). The use of Bt toxins has been widely adapted in cotton and corn for the control for various insects and has already since introduction resulted in the development of resistant pest populations. The proof of concept for the use of Cry proteins has been shown in C. elegans as well as shown reduction in egg production of M. incognita on tomato (Cottage and Urwin, 2013). Use of RNA interference (RNAi) has been investigated more frequently in recent years for functional analysis of plant-parasitic nematode genes. The RNAi process involves the presence of double-stranded RNA that triggers degradation of complementary mRNA resulting in suppression of the specific gene (Cottage and Urwin, 2013). In Europe, there is intolerance to genetically modified crops, while genetically modified crops are common in the US and most planted are genetically modified for herbicide tolerance (Cottage and Urwin, 2013; USDA, 2016). In 2016, 94% of all soybean planted in the United States are genetically modified and of the 94% all are genetically modified for herbicide resistance (USDA, 2016). Use of genetically modified resistance for nematodes is not currently widespread.

Biological control of plant parasitic nematodes can arise through predacious or antagonistic microorganisms, protection of the plant by a microorganism, antibiotic or parasitic action inhibiting the nematode, or beneficial microorganisms increasing the health of the plant (Trivedi, 1992). Predators of plant-parasitic nematodes include other nematodes, mites, insects and other invertebrates. These organisms are essential to maintain in a healthy soil system however application of them as a biological control is impractical. A variety of nematophagous fungi have been analyzed with varying success as methods of control.
(Viaene et al., 2013). Reduction of *M. incognita* soil population has been shown to be significant by *Acremonium strictum* and *Aspergillus terreus* (Singh and Mathur, 2010). Most bacteria present in the rhizosphere that inhibit nematode behavior do so by production of antibiotics, enzymes or toxins (Viaene et al., 2013). Significant planning is needed in use of these as control methods (Trivedi, 1992). Success of application is affected by many components in the process including proper selection of an agent as well as formulation of the agent as a control method (Viaene et al., 2013). Incorporation of a bacterial strain, *Bacillus firmus* I-1582, in the seed coating as well as clothianidin, an insecticide as Poncho ®/ VOTiVO ® (Bayer CropScience, Research Triangle Park) is a biological seed treatment used to control a wide range of nematodes as well as various insects on corn, soybeans, cotton, and sorghum.

Applications of chemical nematicides to soil or plants are often used to manage plant-parasitic nematode populations to below economic damage thresholds. The mode of action may be ‘cidal’ - compounds that when administered are lethal to the nematode, or ‘nematostatic’ and inhibit nematode behavior in some way with reversible affects (Schomaker and Been, 2013). In the global market nematicide use is valued at approximately US$1 billion per year with the majority of this market, 48%, going towards control of root-knot nematodes and 30% going towards control of cyst nematodes. The production of a chemical by a crop protection company typically takes 8-10 years and cost approximately US$60M from initial screening through product launch -- a driving force behind any decision promoting the production of new nematicides.
The most common classification of nematicides is into two groups: fumigants and non-fumigants. Non-fumigants include a wide variety of chemical groups including organophosphates, carbamates, and avermectins (Haydock et al., 2013). Organophosphates and carbamates are nematicidal by binding with acetylcholinesterase (AChE) resulting in inhibition of AChE and build up of acetylcholine which leads to eventual paralysis of the nematode. Nematodes often die due to starvation as they are unable to migrate to a host (Opperman and Chang, 1992). Within non-fumigants, the method of application can be through a liquid method including spray, chemigation or others or through microgranules (Haydock et al., 2013). Examples of liquid formulation nematicides include oxamyl, abamectin and more. Microgranules are a formulation of nematicide that varies in application method based on nematode and crop being treated. Examples of microgranule nematicides include aldicarb and fosthiazate as well as others (Haydock et al., 2013).

Fumigants are compounds based on halogenated hydrocarbons including 1,3-dichloropropene (1,3-D) and methyl bromide which are effective nematicides presumably by inhibiting biochemical pathways while other fumigants are isothiocynate liberators which are presumed to prevent respiration (Haydock et al., 2013). Fumigants began being used in agriculture at the end of the 19th century with carbon disulphide (Haydock et al., 2013). The success of this first fumigant resulted in the development of additional fumigants after the turn of the century including chloropicrin, 1,3-dichloropropene (1,3-D) and methyl bromide. Important characteristics of fumigants include volatility and solubility in order to ensure effectiveness against nematodes. These properties also allow for rapid loss of the chemicals.
into the environment and as a result sometimes require an impenetrable cover. Efficacy of fumigants is based on soil structure, texture, organic matter, moisture content and temperature (Haydock et al., 2013).

A variety of reasons, including their effects on the environment and human safety, have resulted in restriction of use of various nematicides over the years (Haydock et al., 2013). Nematicides are typically non-selective and result in non-target effects through direct ingestion or contact (Haydock et al., 2013). Carbofuran has been shown to be lethal to soil organism such as *Collembola*, beetles and earthworms (Van Straalen and van Rijn, 1998). Contamination of the groundwater can occur from application of nematicides as well as through leaching from the soil to the ground water below. Examples of leaching have been reported over the years with variations on the levels based on soil factors and weather conditions (Oki and Giambelluca, 1989; Karpouzas et al., 2007; van Alphen and Stoorvogel, 2002). Fumigants such as dibromochloropropane (DBCP) and ethylene dibromide (EDB) lost registration in the 1970’s in the United States due to groundwater contamination and carcinogenicity. Methyl bromide was a broad-spectrum fumigant used to control many pests but as a part of the Montreal Protocol on Substance that Deplete the Ozone Layer its use was initially reduced and then eventually stopped. Certain exemptions allow for this use but the product was phased-out by 2005 in developed countries (Haydock et al., 2013).

The loss in use of many nematicides because of environmental and toxicity concerns has prompted the development of a new generation of chemical nematicides that address these concerns and provide effective management of nematodes in agriculture. An example
of this is spirotetramat, a product produced by Bayer CropScience (RTP, NC) labeled as Movento™. Spirotetramat provides control for many crops against nematodes and insects. Vang et al. (2016) showed how timing of application of spirotetramat can be optimized for nematode control. The timing has been determined as significant due to the way in which spirotetramat effects the nematodes, by arresting development (Vang et al., 2016). In addition to spirotetramat, a SDHI fungicide, fluopyram, has recently been demonstrated to reduce nematode damage in crops (Faske and Hurd 2015), but detailed understanding of this observed nematicidal activity is limited.

Properties of Fluopyram

Fluopyram was developed initially as a fungicide by Bayer Crop Science (RTP, NC). Fluopyram is a succinate dehydrogenase inhibitor (SDHI) in the phenyl-benazmide chemical group. The chemical structure of fluopyram is shown in Figure 1. The fungicide resistance action committee (FRAC) code for fluopyram is 7, the group in which all SDHI fungicides fall (Bayer CropScience, pers. comm.). SDHI compounds inhibit cellular respiration of fungi by binding to the complex II or succinate-ubiquinone oxidoreductase in the mitochondria. When a compound is bound to the complex II, ubiquinone cannot bind to the complex inhibiting the ability of the cell to complete the electron transport chain to the production of ATP (Avenot and Michailides, 2010). Other chemicals included in this group are primarily carboxamides, while fluopyram is not. Fluopyram is known to move systemically through foliage acropetally (Bayer CropScience, pers. comm.).
Fluopyram as a Fungicide.

Fluopyram is labeled as three fungicides including Luna Tranquility, Luna Experience, and Luna Sensation (Bayer CropScience, Research Triangle Park, NC). Luna Tranquility®’s formulation includes fluopyram and pyrimethanil (FRAC 9), Luna Experience®’s formulation includes fluopyram and tebuconazole (FRAC 3), and Luna Sensations®’s formulation includes fluopyram and trifloxystrobin (FRAC 11). These three additional fungicides all are broad spectrum fungicides with activity against various fungal pathogens on many crops.

Fluopyram’s activity on various fungi have been critically assessed including its ability to control *Fusarium virguliforme*, the causal agent of sudden death syndrome in soybean. Many isolates of *F. virguliforme* tested by Want et al. (2016) showed sensitivity to fluopyram. Various fungal pathogens have developed resistance to succinate dehydrogenase inhibitor fungicides including boscalid, flutolanil, and carboxin (Amiri et al., 2014; Avenot et al., 2011; Miles et al., 2013). This development in resistance is due to site specificity of binding of the fluopyram molecule to the binding site within cells. Mutation of this binding site allows fungi to overcome fungicide pressure. *Botrytis cinera*, *Didymella bryoniae*, and *Alternaria solani* include fungi that have exhibited resistance to SDHI fungicides (Amiri et al., 2014; Avenot et al. 2011; Miles et al., 2013).

Fluopyram as a Nematicide

Fluopyram is labeled as a nematicide as Velum Prime, Velum Total and as part of the seed treatment ILeVO (Bayer CropScience, Research Triangle Park, NC). Velum Prime ® is
labeled for dozens of crops including broccoli, cabbage, potato, and tomato to name a few, in 31 states in the US. Velum Prime ® is label for use against root-knot nematodes, cyst nematodes, other soil nematodes, white mold, early blight, powdery mildew, and alternaria brown spot. Velum Total ® is a formulation of fluopyram and imidacloprid, an insecticide. Velum Total ® is labeled for cotton and peanut in 15 states against aphid, fusarium, leafhopper, white mold, nematodes, plant bug, early leaf spot, late leaf spot, target leaf spot, thrips and whitefly. ILeVO ® treated seed is currently produced for soybean seed against *Fusarium virguliforme*, the causal agent of sudden death syndrome as well as nematodes in soybean and is available in 36 states.

Recent research has shown that fluopyram can be used as an effective control against *Meloidogyne incognita* and *Rotylenchulus reniformis* (Faske and Hurd, 2015). Exposure of *M. incognita* and *R. reniformis* to a variety of SDHI fungicides including fluopyram show that fluopyram provides the highest level of control. Two-hour EC$_{50}$ values were determined for *M. incognita* and *R. reniformis* to be 5.18 and 12.99 µg/ml fluopyram, respectively (Faske and Hurd, 2015). Recovery of *M. incognita* and *R. reniformis* was more than 50% when exposed to fluopyram for 1 hour. There was a reduction in infection of plants inoculated with nematodes that were exposed to fluopyram for both *M. incognita* and *R. reniformis* (Faske and Hurd, 2015).

**Thesis objectives**

Objectives of this thesis include determining the mode of action of fluopyram on nematodes through use of mutant *C. elegans*. These mutant *C. elegans* have knockdown in
the *sdh1* gene resulting in reduced expression of succinate dehydrogenase, the target of fluopyram in fungi and proposed target in nematodes. This thesis also includes investigation of the effect fluopyram has on nematode eggs and its potential ability to inhibit hatch in *C. elegans, M. incognita,* and *H. glycines.* Control of this life stage of plant-parasitic nematodes can ensure decreased infection in cropping systems. The ability of *C. elegans* and *M. incognita* to recovery after exposure to fluopyram is also investigated here. This is significant to understand in order to ensure sufficient exposure to the compound can be achieved in field settings. The potential effect of soil type on efficacy of fluopyram to decrease egg production of *M. incognita* and *H. glycines* during plant infection is assessed here to determine if potential fluopyram binding to organic matter will affect its ability to control nematode infection of plants.
REFERENCES


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Figure 1. The chemical structure of fluopyram.
IN VITRO EFFECT OF FLUOPYRAM ON NEMATODES

ABSTRACT

Exposure to fluopyram (Velum®, Bayer CropScience, Monheim, Germany) elicits adverse effects on both free-living and plant parasitic nematodes at various life stages. The mode of action of fluopyram, a succinate dehydrogenase inhibitor (SDHI), has been extensively studied on fungi but the mode of its affects against nematodes have been observed but the mode of action is unclear. Exposure of mutant Caenorhabditis elegans with a knockdown in the succinate dehydrogenase gene sdh1, known as VC294, resulted in the EC_{50} of 4.31 ppm compared to wild-type C. elegans EC_{50} of 11.4 ppm, signifying fluopyram works as a SDHI in nematodes. Nematode egg exposure to fluopyram resulted in a significant (p<0.05) decrease in hatch rate in Meloidogyne incognita (southern root-knot nematode) and Heterodera glycines (soybean cyst nematode) however it did not effect C. elegans hatch rate. Recovery from fluopyram exposure did not occur after C. elegans were exposed for 24 hours while recovery did occur when M. incognita were exposed for 1 hour. The lack of registered, safe nematicides in the market make the understanding of the nematicidal effects of fluopyram important to crops that are affected by these pathogens.

INTRODUCTION

Plant parasitic nematodes have been estimated to be responsible from $80 to $125 billion in annual global economic crop losses (Jones et al., 2013; Chitwood, 2003). Root-knot nematodes (Meloidogyne spp.) and cyst nematodes (Heterodera spp. and Globodera spp.) are considered to be the two most important nematode groups due to economic losses caused
from these species (Jones et al., 2013). *Meloidogyne* spp. have been reported to be responsible for the greatest amount of economic damage to agricultural crops due to their wide host range of greater than 2,000 plant species and variety of preferred climates (Sasser and Freckman, 1987). Tomato without the *Mi* resistance gene is considered to have up to 100% damage potential to *M. incognita* and is referred to as the “universal host” (Seid et al., 2015). *Heterodera glycines* is the most significant pest of soybean and causes an estimated US$1 billion in annual crop losses (Liu et al., 2012). Due to their economic importance, the focus of this study was on *M. incognita* and *H. glycines*.

*Meloidogyne incognita* and *H. glycines* hatch from eggs in soil and penetrate plant roots as motile second-stage juveniles (J2). There they establish feeding sites in root vascular tissues and develop to reproductive maturity as sedentary endoparasites. These species of nematode are obligate biotrophs that must feed from live cells and require the parasitism of a host to complete their life cycle. Due to this life style, *M. incognita* and *H. glycines* damage their host plants but rarely to the point of death of the host. Symptoms common in plants during infection of root knot nematode or soybean cyst nematode include stunting, incipient wilt, chlorosis, leaf drop and increased susceptibility to other disease that all contribute to reduced yield (Mitkowski and Abawi, 2003).

Below ground symptoms of the two nematodes differ with the formation of galls or “knots” in the case of *Meloidogyne* spp. infection. These knots are due to the change of normal plant cells to a feeding site known as giant cells by the secretion of effectors by the nematode and hyperplasia of plant cells surrounding the feeding site (Agrios, 2005). Soybean
cyst nematodes similarly modify host cells through effectors, however instead of giant cells syncytia are formed as a feeding site. Formation of a syncytium, a sink for metabolites of the plant that the nematode feeds from, does not result in swelling of the root due to host plant response. Instead, hardened yellow to cream colored cysts can be observed protruding from root tissue. Formation of the cyst from a swollen female’s body wall results in a hardened survival structure for the eggs (Niblack, 2005).

Methods for management of root-knot and cyst nematodes depend upon the cropping system and primarily include host plant resistance, rotations to non-host crops, and nematicides. An effective management strategy for soybean cyst nematodes (SCN) is crop rotation, however the use of non-host is not always economically viable. Soybeans have been bred for resistance to SCN and incorporation of SCN-resistant cultivars with non-host rotations helps extend the durability of resistance, but alternative control strategies for *H. glycines* are still needed (Niblack, 2005). Rotation is often not a viable option for control of *M. incognita* due to its wide host range. The ban of many fumigants, including methyl bromide, as well as many effective organophosphate and carbamate nematicides has resulted in an opportunity in the market for new nematicides with reduced mammalian toxicity and low environmental impact. Use of nematicides to control root-knot and cyst nematodes make up the majority of the US$1 billion global nematicide market with 48% and 30% spent, respectively (Haydock et al., 2013). Chemicals that are labeled for nematode control are either nematicidal or nematostatic, meaning they kill the nematode outright (cidal) or paralyze
the nematode temporarily (static), often leading to death through starvation (Opperman and Chang, 1992).

Fluopyram, currently labeled as a fungicide and nematicide (produced by Bayer Crop Science LP, Monheim, Germany) suppresses cellular respiration. Fluopyram (N-[2-[3-chloro-5-(trifluoromethyl)-2-pyridinyl] ethyl]-2-(trifluoromethyl) benzamide) is a broad-spectrum systemic fungicide of the pyridinyl-ethylbenzamide group. Fluopyram is a succinate dehydrogenase inhibitor (SDHI) that blocks cellular respiration from occurring by binding with cellular succinate dehydrogenase, disrupting the electron transport chain. Use of fluopyram as a nematicide was discovered serendipitously and has been the focus of limited research with root-knot and reniform nematodes (Faske and Hurd, 2015). Use of fluopyram as a seed treatment on soybean seed, as ILeVO®, has been labeled in 36 states however the effect of fluopyram on *H. glycines* has not been extensively researched. The effect of fluopyram on the egg stage of the nematode has not been researched. Fluopyram’s chemical structure (Figure 1) includes three fluorine’s on either end of the compound and is unique from other SDHIs (personal correspondence, Bayer CropScience).

*Caenorhabditis elegans*, a free-living nematode, has been extensively studied and employed as a model organism for studying nematodes and multicellular animals. A comparison between efficacy of nematicides on *C. elegans* and plant parasitic nematodes shows that there are common sensitivities to compounds, making *C. elegans* a good precursory nematode when investigating nematicides (Burns et al., 2015). *C. elegans* eggs are nearly impenetrable by compounds due to selectivity of their inner most layer in the
eggshell (Porta-de-la-Riva, 2012). This layer is the osmotic/permeability barrier for *C. elegans* eggs and the size of its pores determines what enters the organism (Olson et al., 2012). Many natural and induced mutants of *C. elegans* exist, and this study includes *C. elegans* with mutation of the *sdh1* gene, a knockdown of the succinate dehydrogenase (Caenorhabditis Genetic Center (CGC)). In this study, *C. elegans* are used to determine the mode of action of fluopyram on nematodes with the expectation that *sdh1* mutants will elicit an over sensitive response compared to wild type *C. elegans*. The over sensitive response is due to the lower expression of succinate dehydrogenase and the introduction of an inhibitor of that enzyme. A lower concentration will therefore be necessary to elicit a response on an *sdh1* knockdown mutant that non-mutant *C. elegans* which have normal *sdh1* expression levels. Additionally, the influence of fluopyram on *C. elegans, M. incognita, and H. glycines* egg hatch rate was assessed.

The objectives of this study were to determine the effects of fluopyram on hatch of nematode eggs, other nematode life stages, and potential recovery from fluopyram exposure. Ovoid effects were assessed in hatch experiments including *C. elegans, M. incognita, and H. glycines*. The mode of action of fluopyram was investigated by looking at mutant *C. elegans*, with a knockdown of the succinate dehydrogenase gene, *sdh1*. An over sensitive response to and fluopyram of *sdh1* mutant *C. elegans* would signify the mode of action to be a SDHI. The effect of 24-hour exposure to fluopyram followed by recovery was tested with *C. elegans* to determine if the compound works as a nematicide or nematistat.
MATERIALS AND METHODS

Nematode Propagation

The two strains of *C. elegans* used (N2 and the VC294 *sdh1* mutant) were cultured in the same way, on petri plates with nematode growth medium (NGM) and OP50 *E. coli* as a food source. NGM plates were made by autoclaving 3 g of NaCl, 2.5 g Bacto-peptone, and 17 g of agar with 1 L dH2O. After cooling in a water bath to 55°C, 1 mL cholesterol at 5 mg/mL in ethanol, 1 mL 1 M CaCl2, 1 mL 1 M MgSO4, and 25 mL of 1 M KH2PO4 at pH 6.0 are added in the ordered listed. Media is then poured into plates. NGM plates were seeded with overnight culture of OP50 strain of *E. coli* and were placed in 37°C to dry for at least 8 hours. Overnight culture was made by incorporating the OP50 *E. coli* strain, stored in a glycerol stock at -80°C, and LB liquid media and shaking for 12 hours before seeding the plates (Lewis and Fleming, 1995). Plates containing nematodes were kept at room temperature in the dark.

*Meloidogyne incognita* and *Heterodera glycines* were propagated on roots of tomato and soybean plants, respectively, in greenhouse pots. Tomato (*Solanum lycopersicum cv Rutgers*) seedlings were germinated and replanted into clay 6-inch diameter pots with sterile sand while soybean (*Glycine max cv Hutcheson*) seeds were planted directly into 6-inch diameter pots with sterile sand grown in a greenhouse. Second stage juveniles and eggs of race 4 of *M. incognita* were used to inoculate tomato plants and *H. glycines* isolate OP50 were used to inoculate soybean plants of greenhouse cultures. At a minimum of 60 days after inoculation, eggs of *M. incognita* and *H. glycines* were extracted from host plant roots as
described below.

**Nematode Egg Extraction**

*C. elegans* eggs were isolated by collecting mixed stage populations and exposing them to sodium hypochlorite buffer in order to kill all stages except the eggs. Recipe 1 of Porta-de-la-Riva et al. (2012) was used including 2.75 mL dH$_2$O, 1.25 mL of 1 M sodium hydroxide, and 1 mL of 4% sodium hypochlorite. The eggs were pelleted by centrifugation and rinsed of bacteria and debris and concentrated to approximately 1 worm/μl.

The eggs of *M. incognita* were extracted from tomato roots by rinsing roots with 0.5% sodium hypochlorite for 30 seconds (Hussey and Barker, 1973). Roots were massaged by hand to dislodge egg masses and then rinsed with water. The product of extraction was collected with a series of sieves with a 25μm-opening sieve collecting the eggs (Cobb, 1918). The eggs of *H. glycines* were extracted from soybean roots by blasting the cysts off the roots with high-pressure water hose. Cysts were collected with a 250μm-opening sieve. Cysts were gently crushed over the 250μm sieve with a rubber stopper to release eggs that were collected in a 25μm sieve. Eggs collected from *M. incognita* and *H. glycines* were separated from debris by centrifugation in a 70% sucrose suspension (Agrios, 2005).

**Effect on *C. elegans* life stages**

To determine the mode of action of fluopyram on nematodes, mutant VC294 *C. elegans* were exposed to the compound alongside wild-type N2 *C. elegans*. The VC294
strain of *C. elegans* has a knockdown of the *sdh1* gene, a gene involved in the formation of succinate dehydrogenase. Mixed stages of nematodes were rinsed from NGM plates with M9 buffer and concentrated to about 100 nematodes/ 100 μl of M9 buffer. Test medium was made by adding 10 mL of overnight culture, OP50 *E. coli* that was grown for 12 hours in LB broth, to 100 mL of M9 buffer. This test medium was supplemented with 5 mg ampicillin and 2 mg nystatin and stirred, covered at room temperature for 2 hours. 800 μl of test medium was added to each well in 24-well 1 pipetting. The 24-well plates were kept at room temperature and nematodes were observed under a dissecting microscope at initial treatment exposure as well as at 1 hour, 24 hours, 48 hours and after 1 week after exposure. Percentage surviving was determined by observing dead worms as straight, non-moving worms and compared to the total of worms per well. Treatments included technical grade fluopyram at 100, 50, 25, 20, 10, 7.5, 5, 2.5, 1, 0.5 and 0.1 ppm. Positive controls included abamectin (ABM) at 0.5 ppm, spirotetramat-enol (SPT-enol) at 90 ppm (Vang et al., 2016), and mebendazole (MBZ) at 10 ppm, and negative controls included of buffer only and acetone-Triton X-100 solvent used to solubilize fluopyram at a final concentration of 0.1%/well. The positive controls were included to compare the behavior in which fluopyram affects nematode populations. ABM is comprised of avermectin compounds derived from *Streptomyces avermitilis*, a soil bacterium. These compounds act as an acute toxin by targeting the nervous system and paralyze nematodes (Martin et al., 2002). MBZ, a chronic toxin, inhibits synthesis of microtubules in the intestine resulting in the nematodes inability to uptake nutrients (Spence et al., 1982). SPT-enol reduces the nematodes ability to develop
to reproductive maturity (Vang et al., 2016) and is marketed as a nematicide by Bayer CropScience as Movento™. This test was completed once with the N2 wild-type C. elegans and twice more with wild-type and the VC294 mutant C. elegans. Nematode strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440).

**Recovery of C. elegans after 24 hours Fluopyram exposure**

To determine if the effect of fluopyram could be reversed after exposure to the compound was stopped, recovery assays were performed on C. elegans. The recovery assay included suspension of wild-type C. elegans in borate buffer (100mM Na borate, 1mM EDTA, 1mM NaN₃, 1 mg/ml BSA, pH 8.0) and inclusion of the nematodes with treatments into 96-well plates to a volume of 100μl. Mixed life stage C. elegans were exposed to each treatment for 24 hours at which point half of the exposed nematodes underwent a recovery event while the other half remained exposed to the prescribed treatment. Recovery included a triple wash of the nematodes with a borate buffer and then returned to the 96-well plates (Opperman and Chang, 1991). Treatments included fluopyram at 10ppm and ABM at 0.5 ppm. Observations and quantification of living and dead nematodes were made after 24 hours in treatment solution, as well as at 1 hour and 24 hours after recovery. This assay was repeated twice.

**Recovery of M. incognita after Fluopyram exposure**

In addition to C. elegans, recovery assays were performed on M. incognita to determine if the effect of fluopyram could be reversed after exposure to the compound was
stopped. Hatched second-stage juveniles (J2s) of *M. incognita* were incubated in 500 ul of treatment solution for 1 hour then J2 in half of the wells were triple-rinsed in dH2O and allowed to recover for 1 hour and 24 hours. Treatments were replicated five times and included fluopyram at 10 ppm, SPT at 90 ppm and dH2O. Observations and quantification of living and dead nematodes were made after 1 hour exposure to treatment, as well as 1 hour and 24 hours after recovery. This assay was repeated twice.

**Effect on hatch rate of *C. elegans***

To determine the ovoid effect of fluopyram on *C. elegans*, *C. elegans* were synchronized by exposing mixed stage populations to sodium hypochlorite buffer in order to kill all life stages except the eggs. Recipe 1 of Porta-de-la-Riva et al. (2012) was used including 2.75 mL dH2O, 1.25 mL of 1 M sodium hydroxide, and 1 mL of 4% sodium hypochlorite. Eggs were pelleted by centrifugation and rinsed of bacteria and debris and concentrated to approximately 1 worm/µl. Approximately 50 eggs were placed in 24-well plates with a 50 µl treatment and 400 µl M9 buffer for a total of 500 µl. Treatments included 50, 25, 20, 10, 5, 2.5, 1, and 0.5 ppm fluopyram as well as M9 buffer, solvent (acetone-triton), ABM at 0.5 ppm, and SPT-enol at 90 ppm. Plates were stored at room temperature in the dark. After 3 days hatched worms were counted and compared to the initial egg count to determine hatch rate (Porta-de-la-Riva et al., 2012).

**Effect of hatch rate on *M. incognita* and *H. glycines***

A modified Baermann technique (Vang et al. 2016) was used to determine the effect of fluopyram on hatch rate of *M. incognita* and *H. glycines*. In the modified Baermann
technique bowls are used instead of funnels for collection of motile second-stage juveniles that hatch from eggs. A mesh screen fixated to a support, small PVC pipe pieces, was placed in a bowl. The PVC pipe allowed for the support of double layer Kimwipe filter paper on which the eggs are put to hatch. An estimated 10,000 purified eggs of either *M. incognita* or *H. glycines* were applied to the top of Kimwipe filter paper in each bowl (Whitehead and Hemming, 1965). Fluopyram and control treatment solutions were added to the bottom of the bowl so that the treatment just reached to bottom of the screen, allowing egg exposure and movement of hatched J2’s into the treatment solution. Only small bowls of the same size were used to ensure uniformity among replications in each run of the assay.

Treatments for hatch solutions included 50, 35, and 25 ppm fluopyram as well as dH$_2$O and acetone-triton. Hatched nematodes were collected 3, 5, and 7 days after initial exposure. Total nematodes collected per bowl were summed over time and used to determine hatch rate. There were three replications of each treatment per run with three runs of the experiment for both nematode species.

**Statistical Analysis**

To analyze the effect on hatch rates a two-way ANOVA (p<0.05) was run in SAS version 9.4 (SAS Institute, Cary, NC) using a generalized linear model (glm) to compare the data from replications of each assay. When no statistical significance was determined between separate tests so the data from the experiments were combined. The statistical significance of each treatment was determined by comparing using a two-way ANOVA using
the same generalized linear model method. The resulting means were sorted and grouped based on Tukey’s Honest Significant Difference (HSD).

**Dose response curve generation**

The EC\textsubscript{50} relationship between surviving nematodes after 24-hour exposure versus concentration of fluopyram was assessed for wild-type N2 and VC294 mutant *C. elegans*. The dose response curves and EC\textsubscript{50} for each strain were determined through the dose response curves (drc) package in R version 3.3.3 (http://www.r-project.org/) statistical software. This package allows for the fitting of the data, combined between all three assays for each strain, to a three-parameter log-logistic regression model (Ritz et al., 2015).

\[
y = \frac{y}{1 + \exp\left(\frac{\log y - \log y_{50}}{b}\right)}
\]

The variable in this model include x as the fluopyram concentration in ppm, e as the EC\textsubscript{50}, b as the slop around e, and d as the upper-limit of the curve.

**RESULTS**

**Effect on nematode hatch rate**

The hatch rates of the various treatments for *C. elegans* were compared via two-way ANOVA and found that there was not significance between any of the treatments (p<0.05). Comparisons of average hatch rates among the treatments are shown in Figure 1 with the percentage of egg hatch shown for each treatment.
M. incognita and H. glycines were similarly compared via two-way ANOVA and significant reduction in hatch was found between the dH2O untreated control and all the fluopyram treatments. The tests were initially compared against one another and no interaction was found so all similar treatments from each experiment were combined. The average hatch rate of M. incognita is shown in Figure 2 with the percentage of egg hatch compared between the negative controls and fluopyram treatments. The average hatch rate of H. glycines is shown in Figure 3 with the percentage of egg hatch compared between the negative controls and fluopyram treatments. In general, hatch rates of H. glycines were lower than hatch rates of M. incognita and hatch rates of both species significantly decreased with increase in fluopyram concentration.

Effect of exposure to fluopyram on C. elegans life stages

Dose response curves were generated for wild-type N2 and mutant VC294 C. elegans and are shown in Figures 5 and 6 respectively. VC294 C. elegans (sdh1) mutants showed an over sensitive response to fluopyram when exposed to the same concentrations as wild-type N2 C. elegans. This is shown through the EC50 of VC294 of 4.31 ppm compared to the wild-type’s EC50 of 11.4 ppm. N2 and VC294 C. elegans not exposed to fluopyram after 24 hours (Fig. 7-8) had average survival rates of 95% and 94% respectively. C. elegans exposure to ABM resulted in a 24 hour average survival rate of 5% for N2 and 1.7% for VC294.

Recovery of C. elegans after 24 hours Fluopyram exposure

C. elegans exposure to 10ppm fluopyram for 24 hours followed by recovery resulted in low survival rates signifying recovery is not occurring (Fig. 9). Number of dead nematodes
and total number of nematodes were used to calculate the percentage of surviving or live nematodes. After 24 hours the percentage of live nematodes decreased to 1.96% and 4% in the two repetitions, respectively. Within 48 hours the surviving nematode population had dropped to 1.6% and 0%, in the two repetitions, respectively. Figure 9 shows the populations of surviving nematodes in the treatments after 24 hours of recovery. The recovered nematodes are compared to non-recovered or continuously exposed nematodes with each treatment.

Two-factor ANOVA did not indicate any significant (p<0.05) experiment by treatment interaction so the data from independent tests were combine. One-factor ANOVA of fluopyram treated nematodes indicated there was no significance (p<0.05) between treatment type (recovered or continuously exposed), shown in the first two bars of Figure 9. One-factor ANOVA of ABM treated nematodes indicated there was no significance (p<0.05) between treatment type (recovered or continuously exposed), shown in the third and fourth bars of Figure 9.

**Recovery of *M. incognita* after Fluopyram exposure**

*M. incognita* exposure to 10ppm fluopyram for 1 hour followed by recovery resulted in high survival rates. Survival rates for the three treatments after 24 hours of recovery are shown in figure 9 and are expressed in percentage of live worms. In repeated tests, 1-hour exposure to fluopyram resulted in recovery rates of 84% and 90%, respectively, after 24 hours’ recovery period. This was similar to the survival rates of SPT and dH2O, both of which are expected to have little to no negative effect on long term survival rate due to the
mode of action, arrest development, of SPT (Vang et al., 2016) and lack of nematicide in dH$_2$O.

Two-factor ANOVA did not indicate any significant (p<0.05) experiment by treatment interaction so the data from independent tests were combine. One-factor ANOVA treated nematodes indicated there was not a significant (p<0.05) interaction between treatment, shown in Figure 10.

**DISCUSSION**

Fluopyram was lethal to mixed life stages of *C. elegans*, and this effect increased as the concentration of fluopyram increased. The VC294 mutant of *C. elegans* had a lower LD$_{50}$ than that of wild-type *C. elegans* signifying an over sensitive response to fluopyram treatment over a 24-hour period. The exposure of a mutant with a knockdown of the *sdh1* gene to an SDHI-inhibitor would result in an over sensitive response if the succinate dehydrogenase was the target of the compound. This demonstrates the mode of action of fluopyram to be a succinate dehydrogenase inhibitor in nematodes, as previously concluded in fungi. Other SDHI fungicides have been tested as nematicides with little success but the ability of fluopyram to control nematodes is likely due to the chemical structure of the compound (Faske and Hurd, 2015). It is hypothesized the three fluorine’s, small in size, on both ends allow the compound to bind in smaller binding locations on enzymes and result in greater affinity for the molecule to block cellular respiration.
The highly selective permeability of the eggshell of *C. elegans* could be responsible for limiting the affect of fluopyram on hatch rate. The inability of fluopyram to enter the egg and kill the embryo before hatching as an L1 nematode resulted in no significant difference between hatch rates among all treatments. In particular, the innermost layer is the osmotic and permeability barrier for the *C. elegans* embryo. This barrier is nearly impenetrable to small molecules (Olson et al., 2012). Fluopyram successfully inhibited the hatch of *M. incognita* and *H. glycines* eggs but did not have an effect on *C. elegans* hatch rate. The results shown provide evidence for the ability of fluopyram to enter the egg of *M. incognita* and *H. glycines* and stop the nematode from hatching by killing the embryo, J1, or J2 stage of the nematode while it alternatively cannot penetrate the innermost layer of *C. elegans* eggshell (Olson et al., 2012) and therefore does not inhibit hatch.

The inability of *C. elegans* to recovery after 24 hour fluopyram exposure is evident in the low survival rates after 24 and 48 hour recovery periods, the highest being 4%. The effect of *M. incognita* to 1-hour exposure to fluopyram resulted in high survival rates when assessed 24 hours after recovery. This indicates that duration of exposure to fluopyram in the field is critical to suppression of the pathogen. The inability of *C. elegans* to to recover after 24 hour exposure to fluopyram suggests a potential irreversible ability of fluopyram to control nematode populations.

In terms of nematode control in an agricultural setting, the abilities of this compound are an essential tool in the control of plant parasitic nematodes. While control of all stages of a pathogen is the ideal for an active ingredient, control of the infective stage, J2 in the case of
plant parasitic nematodes, is a key characteristic to effective control methods. The ability of fluopyram to penetrate and inhibit egg hatch of plant-parasitic nematodes, as well as kill motile nematode stages, including *C. elegans*, makes it a useful nematicide for agricultural use.
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Figure 2. The average hatch rates of wild-type *C. elegans* after 72 hours exposure to fluopyram. Treatments included various concentrations of fluopyram (50-0.5 ppm). ABM (0.5 ppm) and SPT-enol (90 ppm) were included and saw similar rates of hatch compared to all concentrations of fluopyram. Negative control off M9 buffer used to concentrate nematodes and solvent (0.1% acetone-triton) were included and there was no significant difference between any of the treatments when assessed in a two-way ANOVA with p<0.05 and are grouped with Tukey HSD.
Figure 3. The average hatch rate for *M. incognita* after 1 week exposure to fluopyram. Three fluopyram treatments were included and all resulted in significantly lower hatch rates than negative control of dH2O. Solvent (0.1% acetone-triton) control was included as well and hatch rates were averaged for all treatments over the three repetitions of the experiment. There was a significant decrease in hatch rate between treatments when assessed in a two-way ANOVA with p<0.05 and are grouped with Tukey HSD.
Figure 4. The average hatch rate for *H. glycines* after 1 week exposure to fluopyram. Three fluopyram treatments were included and all resulted in significantly lower hatch rates than negative control of dH2O. Solvent (0.1% acetone-triton) control was included as well and hatch rates were averaged for all treatments over the three repetitions of the experiment. There was a significant decrease in hatch rate between treatments when assessed in a two-way ANOVA with p<0.05 and are grouped with Tukey HSD.
Figure 5. Dose response curve for wild-type N2 *C. elegans* after 24 hours exposure to fluopyram. The curve, generated using R with the drc package, was used to determine the EC$_{50}$ for wild-type N2 *C. elegans* to be 11.4 ppm fluopyram.
Figure 6. Dose response curve for VC294 mutant *C. elegans* after 24 hours exposure to fluopyram. The curve, generated using R with the drc package, was used to determine the EC$_{50}$ for VC294 mutant *C. elegans* which have a knockdown of the *sdh1* gene, to be 4.31 ppm.
Figure 7. Survival of N2 C. elegans after 24 hours exposure to fluopyram. Nematodes were exposed to treatment with the inclusion of a food source.

Figure 8. Survival of the VC294 C. elegans after 24 hour exposure to fluopyram. Nematodes were exposed to treatment with the inclusion of a food source.
Figure 9. Recovery of *C. elegans* after 24 hour exposure to fluopyram. Nematodes were exposed to treatments for 24 hours and then recovered and assessed for survival 24 hours later. The treatments labeled “continued exposure” did not undergo a recovery and remained within the treatment continuously. Treatments included fluopyram at 10 ppm and ABM at 0.5 ppm with recovery and continued exposure for each treatment. Experiments were combined due to lack of significant (p<0.05) interaction between experiment and treatment.
Figure 10. Recovery of *M. incognita* after 1 hour exposure to treatment.

Nematodes were exposed to treatments for 1 hour and then recovered and assessed for survival 24 hours later. Treatments included fluopyram at 10 ppm, ABM at 0.5 ppm, and dH₂O. Experiments were combined due to lack of significant (p<0.05) interaction between experiment and treatment.
IN PLANTA EFFECTS OF FLUOPYRAM ON NEMATODES

ABSTRACT

In chapter one it was shown that fluopyram treatment of plant-parasitic nematodes resulted in decreased hatch rate and infective juvenile survival. Potential effects of fluopyram treatment on nematode infection of host plant roots were assessed in this chapter. Potential effect of soil type on ability of fluopyram to control nematode infection of plants was also assessed. Fluopyram decreased nematode eggs per gram of root when applied to soil containing plants that were inoculated with either *Meloidogyne incognita* or *Heterodera glycines*. The type of soil did not affect the ability of fluopyram to control nematode egg production in inoculated plants; under all soil types tested there was a statistically significant difference (p<0.05) in egg production. Fluopyram has exhibited control of nematode populations in vitro and in planta.

INTRODUCTION

Plant parasitic nematodes have been estimated to be responsible from $80 to $125 billion in annual global economic crop losses (Jones et al., 2013; Chitwood, 2003). Root-knot nematodes (*Meloidogyne* spp.) and cyst nematodes (*Heterodera* spp. and *Globodera* spp.) are considered to be the two most important nematode groups due to economic losses caused from these species (Jones et al., 2013). *Meloidogyne* spp. have been reported to be responsible for the greatest amount of economic damage to agricultural crops due to their wide host range of greater than 2,000 plant species and variety of preferred climates (Sasser
and Freckman, 1987). Tomato without the Mi resistance gene is considered to have up to 100% damage potential to *M. incognita* and is referred to as the “universal host” (Seid et al., 2015). *Heterodera glycines* is the most significant pest of soybean and causes an estimated US$1 billion in annual crop losses (Liu et al., 2012). Due to their economic importance, the focus of this study was on *M. incognita* and *H. glycines*.

*Meloidogyne incognita* and *H. glycines* hatch from eggs in soil and penetrate plant roots as motile second-stage juveniles (J2). There they establish feeding sites in root vascular tissues and develop to reproductive maturity as sedentary endoparasites. These species of nematode are obligate biotrophs that must feed from live cells and require the parasitism of a host to complete their life cycle. Due to this life style, *M. incognita* and *H. glycines* damage their host plants but rarely to the point of death of the host. Symptoms common in plants during infection of root knot nematode or soybean cyst nematode include stunting, incipient wilt, chlorosis, leaf drop and increased susceptibility to other disease that all contribute to reduced yield (Mitkowski and Abawi, 2003).

Below ground symptoms of the two nematodes differ with the formation of galls or “knots” in the case of *Meloidogyne* spp. infection. These knots are due to the change of normal plant cells to a feeding site known as giant cells by the secretion of effectors by the nematode and hyperplasia of plant cells surrounding the feeding site (Agrios, 2005). Soybean cyst nematodes similarly modify host cells through effectors, however instead of giant cells syncytia are formed as a feeding site. Formation of a syncytium, a sink for metabolites of the plant that the nematode feeds from, does not result in swelling of the root due to host plant
response. Instead, hardened yellow to cream colored cysts can be observed protruding from root tissue. Formation of the cyst from a swollen female’s body wall results in a hardened survival structure for the eggs (Niblack, 2005).

Methods for management of root-knot and cyst nematodes depend upon the cropping system and primarily include host plant resistance, rotations to non-host crops, and nematicides. An effective management strategy for soybean cyst nematodes (SCN) is crop rotation, however the use of non-host is not always economically viable. Soybeans have been bred for resistance to SCN and incorporation of SCN-resistant cultivars with non-host rotations helps extend the durability of resistance, but alternative control strategies for *H. glycines* are still needed (Niblack, 2005). Rotation is often not a viable option for control of *M. incognita* due to its wide host range. The ban of many fumigants, including methyl bromide, as well as many effective organophosphate and carbamate nematicides has resulted in an opportunity in the market for new nematicides with reduced mammalian toxicity and low environmental impact. Use of nematicides to control root-knot and cyst nematodes make up the majority of the US$1 billion global nematicide market with 48% and 30% spent, respectively (Haydock et al., 2013). Chemicals that are labeled for nematode control are either nematicidal or nematistatic, meaning they kill the nematode outright (cidal) or paralyze the nematode temporarily (static), often leading to death through starvation (Opperman and Chang, 1992).

Fluopyram, currently labeled as a fungicide and nematicide (produced by Bayer Crop Science LP, Monheim, Germany) suppresses cellular respiration. Fluopyram (N-[2-[3-
chloro-5-(trifluoromethyl)-2-pyridinyl[ethyl]-2-(trifluoromethyl) benzamide) is a broad-spectrum systemic fungicide of the pyridinyl-ethylbenzamide group. Fluopyram is a succinate dehydrogenase inhibitor (SDHI) that blocks cellular respiration from occurring by binding with cellular succinate dehydrogenase, disrupting the electron transport chain. Use of fluopyram as a nematicide was discovered serendipitously and has been the focus of limited research with root-knot and reniform nematodes (Faske and Hurd, 2015). Use of fluopyram as a seed treatment on soybean seed, as ILeVO®, has been labeled in 36 states however the effect of fluopyram on H. glycines has not been extensively researched. The effect of fluopyram on the egg stage of the nematode has not been researched. Fluopyram’s chemical structure (Figure 1) includes three fluorine’s on either end of the compound and is unique from other SDHIs (personal correspondence, Bayer CropScience).

Chapter 1 of this thesis demonstrated that egg hatch of M. incognita and H. glycines was significantly decreased as exposure to fluopyram concentrations increased. It was also demonstrated that fluopyram appeared to be lethal to mixed life stages of Caenorhabditis elegans, and the mode of action, SDHI, was confirmed through observing an over sensitive response in sdh1 knockdown mutant C. elegans. It was also demonstrated that recovery of C. elegans does not occur after 24 hour exposure but recovery did occur in M. incognita exposed to fluopyram for 1 hour.

The objective of this study was to assess the ability of fluopyram to control M. incognita infection of tomato roots and H. glycines infection of soybean roots in greenhouse pot trials in three different soil types. Fluopyram’s high affinity to bind to organic matter may
result in decreased control of nematode populations in soils with higher levels of organic matter.

MATERIALS AND METHODS

Nematode Propagation

_Meloidogyne incognita_ and _Heterodera glycines_ were propagated on roots of tomato and soybean plants, respectively, in greenhouse pots. Tomato (_Solanum lycopersicum_ cv Rutgers) seedlings were germinated and replanted into clay 6-inch diameter pots with sterile sand while soybean (_Glycine max_ cv Hutcheson) seeds were planted directly into 6-inch diameter pots with sterile sand grown in a greenhouse. Second stage juveniles and eggs of race 4 of _M. incognita_ were used to inoculate tomato plants and _H. glycines_ isolate OP50 were used to inoculate soybean plants of greenhouse cultures. At a minimum of 60 days after inoculation, eggs of _M. incognita_ and _H. glycines_ were extracted from host plant roots as described below.

Nematode Egg Extraction and Hatch

The eggs of the _M. incognita_ were extracted from tomato roots by rinsing roots with 0.5% sodium hypochlorite for 30 seconds (Hussey and Barker, 1973). Roots were massaged by hand to dislodge egg masses and then rinsed with water. The product of extraction was collected with a series of sieves with a 25μm-opening sieve collecting the eggs (Cobb, 1918). The eggs of _H. glycines_ were extracted from soybean roots by blasting the cysts off the roots with high-pressure water hose. Cysts were collected with a 250μm-opening sieve. Cysts were
gently crushed over the 250µm sieve with a rubber stopper to release eggs that were collected in a 25µm sieve. Eggs collected from *M. incognita* and *H. glycines* were separated from debris by centrifugation in a 70% sucrose suspension (Agrios, 2005).

Eggs were hatched by modified Baerman method with bowls instead of funnels. Mesh screens supported by PVC pipe pieces were layer with two Kimwipes™ and purified eggs were applied to the filter paper. dH₂O was added to the bottom of the bowl to the point that it touched the filter paper to allow J2 movement into bowl. Bowls were sealed with foil and placed in 28°C growth chambers (Whitehead and Hemming, 1965). *M. incognita* were collected after 3 days in the growth chamber and then refilled with dH₂O and returned to the growth chamber to continue hatching. After the first collection, repeated collections of *M. incognita* occurred every two days. *H. glycines* were collected every two days for approximately a week. Collected J2s were centrifuged and re-suspended to the needed concentrations.

**Effect of Fluopyram on plant Infection by *M. incognita* and *H. glycines***

Plants were grown in three different soil types and each soil type had three different treatments resulting in a total combination of nine different treatment soil type combinations with either 10 per combination in test 1 and 2 and 8 replicates per combination in test 3. The soil types tested included sand, sand and soil combination at a 1:1 ratio, and potting medium. Sand used in all sand pots and in sand:soil combo was pure sterile sand. Soil used in sand:soil combo was sterile soil. Potting medium was Fafard® 4P Mix (Sungro Horticulture, Bellevue,
WA, USA) consisting of Canadian sphagnum peat moss, bark, vermiculite, perlite, dolomitic limestone and wetting agent.

Treatments were water treatment, fluopyram in the formulation Velum Prime® (Bayer CropScience) at the labeled rate of 249.14 g ai/ha (6.84-fl oz/acre), and SPT as the formulation Movento™ (Bayer CropScience) plus adjuvant at the labeled rate of 87.6 g ai/ha (5-fl oz/acre). When plants were 10 cm tall, all plants were inoculated with 10,000 eggs of the appropriate nematode. Rutgers tomato was used for M. incognita infection assays and Hutcheson soybean was used for H. glycines infection assays. Fluopyram treatments were made two days after inoculation by spray to the soil surface in all tests. SPT treatments were made via foliar application until drip (Vang et al., 2016) two days after inoculation in test 1 and two weeks after inoculation in tests 2 and 3.

Plants were grown for two months after inoculation before data collection. Assessments included measuring the above ground height of the plant, measuring the weight of the root system, number of egg masses present on roots in tomato, number of cysts collected from soybean, and number of eggs collected from extraction. Eggs were extracted as mentioned in “Egg extraction and hatch”, with a modification for M. incognita extraction in order to assess egg mass quantity. Tomato roots were washed free of soil and submerged in aqueous Phloxine B at 0.3g/L dH₂O for 30 seconds to stain egg masses red. After exposure to Phloxine B, a sample of the root was counted for the presence of egg masses for each tomato plant. Once egg mass counts were made eggs were extracted with the roots being submerged in a 0.5% sodium hydrochloride solution for 30 seconds while the roots were
massaged by hand in the solution to break away egg masses off the roots and ensure dissolve of the gelatinous matrix. The resulting liquid was poured into 25 µm-opening sieve so eggs would be collected and rinsed into 50 mL tubes.

For *H. glycines* the cysts were collected by blasting the roots with high pressure water over nested sieves and then collected in 50 mL tubes. After cysts were counted they were gently crushed over a 60 mesh, or 250 µm, sieve with a rubber stopper and eggs were collected below in a 25 µm-opening sieve. Eggs were counted on an inverted microscope the same way for both species using three subsamples per root system.

**Statistical Analysis**

In analyzing reversible effects in *M. incognita*, the results from the repetitions of the experiment were compared against each other using a two-way ANOVA (p<0.05) in SAS version 9.4 (SAS Institute, Cary, NC) using a generalized linear model (glm). There was no significant difference between the replications of the assay so the resulting data were combined significance between treatments were assessed using two-way ANOVA using a generalized linear model. The resulting means were sorted and grouped based on Tukey’s Honest Significant Difference (HSD).

In analyzing the infection of plants each repetition was treated as a block and compared against treatment within each soil type. This comparison was made by a two-way ANOVA (p<0.05) in SAS using a generalized linear model (glm) to compare the data from replications of each assay. The resulting means were sorted and grouped based on Tukey’s HSD.
RESULTS

Effect of Fluopyram on plant infection *M. incognita* and *H. glycines*

The level of plant infection by nematodes was measured by eggs per gram of root. Initial assessment of the data across 3 tests showed large variation between soil type and as a result all treatments were analyzed based on soil type separately. For *M. incognita* in sand and potting medium (Figures 10-11) there was a significant difference (p<0.05) between the untreated control and fluopyram treatment in two replications of the experiment but not in a third. For sand:soil combo (Figure 12) there was a significant difference (p<0.05) between untreated control and fluopyram treatment in all three replications. In almost all *M. incognita* tests, egg production after fluopyram treatment was significantly less than the untreated control, regardless of soil type. The significance of *M. incognita* egg reduction by SPT treatment varied among soil types and experiments.

For *H. glycines* in sand (Fig. 13) and sand:soil combo (Fig. 14) there was significant difference (p<0.05) between untreated control and fluopyram treatment in two of the three tests. In potting medium (Fig. 15) there was a significant difference (p<0.05) between untreated control and fluopyram treatment in one of three tests. In *H. glycines* tests that had measurable infection in untreated controls, egg production after fluopyram treatment was significantly less than the untreated control. The significance of *H. glycines* egg reduction by SPT treatment varied among soil types and experiments.
DISCUSSION

Significant reduction in *M. incognita* infection was observed in plants grown for two months after fluopyram treatment. Since significant reduction in *M. incognita* infection compared to untreated controls occurred in at least two out of three tests per soil type, soil type may not be a primary affector for fluopyram activity against root-knot nematodes. This needs to be further tested in soils with higher percentage clay content to ensure that potential soil adsorption does not influence fluopyram activity against nematodes. The high organic matter composition of the potting soil did not appear to adversely affect fluopyram activity, and may reflect the application procedure used to apply fluopyram. SPT also reduced *M. incognita* levels compared to untreated controls in all soil types, but not at the level or consistency of fluopyram across all tests.

Significant reduction in *H. glycines* infection from fluopyram treatment compared to untreated controls was observed in two out of three tests using sand and sand:soil media. Infection of soybean roots by *H. glycines* in potting soil even in untreated controls was generally low, making reductions from fluopyram treatment difficult to determine. The reductions in *H. glycines* infection were most pronounced in sand, which happened to be an optimal medium for infection in the untreated controls.

Compared to the other nematicide, SPT, fluopyram constantly performed as well, and usually better in suppressing nematode infection of plant roots. This may be due to the apparent contact activity of fluopyram (Chapter 1) versus the developmental and systemic activity required for SPT activity (Vang et al., 2016). With one exception, the instances in
which SPT control was not significantly different from the untreated control was in the first experiment in which the timing of application was too close to the time of inoculation to observe a chronic affect (Vang et al., 2016). In general, the egg population of nematodes treated with fluopyram were lower than those of treated with SPT. Combined with the ovicidal properties discussed in Chapter 1, fluopyram demonstrates promising ability to control nematode populations that would be present in agricultural settings.
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**Figure 11. Average *M. incognita* eggs per gram of root in potting medium.** Each treatment is represented by a different color with the treatments including fluopyram, SPT, and untreated control (UTC). The average eggs/g root in plants treated with fluopyram is significantly different from the untreated control in two of the three experiments.
Figure 12. Average *M. incognita* eggs per gram of root in sand. Each treatment is represented by a different color with the treatments including fluopyram, SPT, and untreated control (UTC). The average eggs/g root in plants treated with fluopyram is significantly different from the untreated control in two of the three experiments.
Figure 13. Average *M. incognita* eggs per gram of root in Sand:Soil Combo. Each treatment is represented by a different color with the treatments including fluopyram, SPT, and untreated control (UTC). The average eggs/g root in plants treated with fluopyram is significantly different from the untreated control in all three experiments.
Figure 14. Average *H. glycines* eggs per gram of root in Potting Medium. Each treatment is represented by a different color with the treatments including fluopyram, SPT, and untreated control (UTC). The average eggs/g root in plants treated with fluopyram is significantly different from the untreated control in one of the three experiments.
Figure 15. Average *H. glycines* eggs per gram of root in Sand. Each treatment is represented by a different color with the treatments including fluopyram, SPT, and untreated control (UTC). The average eggs/g root in plants treated with fluopyram is significantly different from the untreated control in two of the three experiments.
Figure 16. Average *H. glycines* eggs per gram of root in Sand:Soil Combo. Each treatment is represented by a different color with the treatments including fluopyram, SPT, and untreated control (UTC). The average eggs/g root in plants treated with fluopyram is significantly different from the untreated control in two of the three experiments.