

ABSTRACT

VANG, LEAH ELSEA. The Effects of Spirotetramat on Nematodes. (Under the direction of Eric Davis.)

Thousands of nematode species are known to parasitize plants worldwide, causing a variety of plant diseases, and resulting in devastating crop losses annually. A primary means of nematode control has been chemical nematicides. Many previously registered nematicides have been banned or restricted in recent years due to their inherent toxicity and environmental hazards. Therefore, there is strong need for registration of safe, effective nematicides. A promising candidate is spirotetramat (Movento™), an effective insecticide that has also been shown to suppress nematode populations. Spirotetramat (SPT) and its metabolites have favorable ecotoxicological and toxicological profiles. Inside the plant, SPT is hydrolyzed to the active enol form which functions as a lipid biosynthesis inhibitor. The enol form is also unique in having acropetal and basipetal systemic movement in plants, providing great potential to affect nematodes that infect plant roots. Little is actually known about how SPT suppresses nematode populations; therefore, the first research objective was to determine what nematode life stages are most affected by SPT-enol. Hatching tests were conducted with the model nematode *Caenorhabditis elegans*, with no significant ($p < 0.05$) effects on hatching rates observed at a maximum SPT-enol concentration of 105ppm. SPT-enol also did not affect hatching of plant-parasitic *Meloidogyne incognita* (southern root-knot nematode) and *Heterodera glycines* (soybean cyst nematode). Life-stage assays conducted with *C. elegans* indicated an arrest of juvenile development before reaching adulthood at SPT-enol concentrations as low as 30ppm ($p < 0.05$). *C. elegans* dose response curves estimated the SPT-enol EC_{95} to be between 44 and 48ppm. Objective 2 evaluated the optimal

time to spray SPT on plant foliage for systemic effects on nematodes infecting the plant's roots. Movento 240SC was applied to plant foliage at the labeled insecticidal rate of 87.6 g ai/ha (5 fl oz/acre) in the greenhouse at 1-week intervals starting at 1 week prior to inoculation with *H. glycines* (soybean plants) or *M. incognita* (tomato plants) and ending applications at 3 weeks after inoculation. Overall, results indicated that SPT inhibits nematode development to reproductive maturity most effectively when applied to host plant foliage at 1-2 weeks after inoculation. Optimal SPT application timings coincide with the early stages of root infection, when nematodes are still in the vulnerable juvenile stage.

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The Effects of Spirotetramat on Nematodes

by
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DEDICATION

I wish to dedicate this document to my husband Jeremy Vang. Thank you for your love and support during this challenging process.

BIOGRAPHY

Leah Vang was born on August 16, 1991 in High Point, North Carolina. She completed her Bachelor of Science degree in Plant Biology from North Carolina State University in 2012. In the fall of 2013, Leah accepted a graduate research program at North Carolina State University in the Department of Plant Pathology to pursue her Master of Science. She was awarded the Bayer CropScience Fellowship in Plant Pathology to research the effects of spirotetramat on nematodes.

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LITERATURE REVIEW

INTRODUCTION TO NEMATODES

Nematodes are aquatic roundworms belonging to the phylum Nematoda. Adapted to virtually every habitat on earth, nematodes are the most abundant Metazoa on the planet (Decraemer and Hunt, 2013). Although they are aquatic organisms, the ability of many nematode species to survive periods of drought in an anhydrobiotic state has allowed them to inhabit even the most unlikely environments, including arid deserts. Nematodes have diverse feeding habits. Some live freely in the soil or bodies of water and feed on bacteria or other microbes, while others parasitize plants and animals. Despite their diversity, all nematodes share a basic life cycle that includes 6 different life stages (egg, four juvenile stages, and adult) separated by 4 molts (Agrios, 2005).

CAENORHABDITIS ELEGANS AS A DEVELOPMENTAL MODEL

In 1963, Sydney Brenner chose to work with a small (roughly 1-mm in length) free-living soil nematode, *Caenorhabditis elegans*, as a model organism (Brenner, 1974). *C. elegans*' small size, short life cycle, hermaphroditic reproduction, ease of genetic manipulation, and large reproductive capacity make it amenable to laboratory studies. It can easily be cultured on agar plates seeded with the bacteria, *Escherichia coli* (strain OP50) and its transparent body allows its anatomical features to be observed under a microscope. In 1998, *C. elegans* became the first multicellular organism to have its genome fully sequenced (*C. elegans* Sequencing Consortium, 1998). Over the past forty years a wealth of knowledge on *C. elegans* biology has been generated and is currently available through many online

resources such as Wormbase (<http://www.wormbase.org>) and Wormbook (<http://www.wormbook.org>). *C. elegans* has been especially valuable as a model for highly conserved biological processes such as development, behavior, molting, and reproduction for plant-parasitic nematodes due to the challenges of using plant-parasitic nematodes in laboratory studies (Jones et al., 2011). As obligate biotrophs with parasitic life cycles that can take several weeks to months to complete, it can be quite difficult to manipulate and obtain specimens of plant-parasitic nematodes for study. Furthermore, trying to observe the development of plant-parasitic nematodes is a daunting task, especially for the sedentary endoparasites such as root-knot and soybean cyst nematodes, which remain inside host plant roots for a majority of their life cycles.

C. elegans is mainly a hermaphroditic species, with self-fertile adult hermaphrodites able to produce up to 350 eggs (Jones et al., 2011). Hermaphrodites contain two XX chromosomes; however, on rare occasions, males can be produced by spontaneous meiotic nondisjunction and contain only one X chromosome. Males can cross-fertilize with the hermaphrodites to enable genetic analyses. Eggs are continuously fertilized by the adult hermaphrodites and laid over a period of about 5 days. The first larval stage (L1) develops inside the egg and hatches. As the larvae grow they progress through 4 molts until they become adults. The entire life cycle takes about 3 days to complete at 25°C, but may take longer as the temperature is decreased. Under optimal growth conditions, *C. elegans* can live for 2-3 weeks. However, under conditions of crowding or starvation the adults start to die off and certain larval stages develop into an alternate third larval survival stage, called a dauer.

Dauers have impermeable cuticles and sealed mouths and are capable of surviving in an arrested inactive state for several months until growth conditions become favorable, at which point they resume normal development (Lewis and Fleming, 1995). The entire life cycle is represented in Figure 1.

INTRODUCTION TO PLANT-PARASITIC NEMATODES

More than 4,000 species of nematodes are known to parasitize plants worldwide, causing massive amounts of crop damage every year (Decraemer and Hunt, 2013). Adaptations for plant-parasitism by nematodes include a hollow protrusible mouth spear (stylet), which is used to pierce plant tissues and withdraw nutrients from plant cells, and enlarged esophageal gland cells that secrete proteins and other small molecules, called effectors, through the stylet and into the plant. Evidence indicates that secreted nematode effectors can modify the structure and function of host cells, but the identities and functions of many nematode effectors remain largely unknown (Mitchum et al., 2013). Most plant-parasitic nematode species feed on plant root cells, siphoning nutrients from the plant and often causing severe root damage or distortion that can lead to above ground symptoms of nutrient deficiency, water stress, and ultimately reduced quality and/or yield.

Associations of some nematodes with other plant pathogens can lead to even further damage. For example, wounds caused by nematode feeding can serve as infection courts for microbial pathogens. Co-infection with nematodes can increase the severity of some fungal diseases. A few nematode species can even vector plant viruses (Agrios, 2005).

A global survey of nematologists in 1985 estimated that the annual economic losses in 21 major crops caused by plant-parasitic nematodes was around \$77 billion (Sasser and Freckman, 1987). In 2003, accounting for inflation, estimated annual crop losses to nematode damage was adjusted to \$125 billion (Chitwood, 2003). Of all the plant-parasitic nematodes, root-knot nematodes (*Meloidogyne* spp.) are considered to be the most damaging and of highest economic importance, infecting more than 2,000 species of plants worldwide (Sasser and Freckman, 1987; Mitkowski and Abawi, 2003). Cyst nematodes (*Heterodera* and *Globodera* spp.) are second in agricultural importance. While species of cyst nematodes are fairly host specific, different species attack many economic crops throughout the world. The soybean cyst nematode (*Heterodera glycines*) is considered to be the leading pathogen of soybean worldwide (Sasser and Freckman, 1987; Davis and Tylka, 2000).

LIFE CYCLE OF *MELOIDOGYNE* SPP.

Root-knot nematodes (*Meloidogyne* spp.) are the number one economic nematode pathogen of crops around the globe (Sasser and Freckman, 1987). The International *Meloidogyne* Project (Sasser et al., 1983) revealed the most abundant species globally to be *M. incognita* (Southern root-knot nematode), *M. javanica* (Javanese root-knot nematode), *M. arenaria* (Peanut root-knot nematode), and *M. hapla* (Northern root-knot nematode). Together, these four species of root-knot nematode account for approximately 5% of annual global crop losses from all sources (Sasser et al., 1983). The diagnostic symptoms of root-knot nematodes are the intercalary galls or “knots” that form on infected roots. Above ground

symptoms can appear as water stress and nutrient deficiency, visible as yellowing, wilting, stunting, and even plant death (Karssen et al., 2013).

Root-knot nematodes (RKN) are sedentary endoparasites, spending a majority of their life cycle embedded within plant roots (Figure 2). Unlike *C. elegans*, the first molt from the first juvenile stage (J1) to J2 stage (plant nematologists use the term juvenile instead of larvae) occurs inside the egg in all plant-parasitic nematode species. Upon hatching, the J2 migrates in soil toward host roots, guided by a gradient of exudates from host roots detected by their chemosensillae (Curtis, 2008). RKN J2s usually penetrate plant roots near the tip, behind the root cap. They then migrate intercellularly toward root vascular tissues in the zone of elongation where they use effectors to transform selected root vascular cells into a permanent feeding site composed of a small group of “giant–cells”. During the time in which giant-cells are formed, surrounding tissues undergo hyperplasia and hypertrophy, forming characteristic root galls that surround the RKN infection sites. Following establishment of a feeding site, J2s progress through a series of molts and become swollen and flask-shaped. During the relatively rapid J3 and J4 life stages, RKN feeding is paused. Feeding by the adult female resumes upon completion of the 4th molt. Many *Meloidogyne* species are parthenogenic, with males unnecessary for reproduction. However, when environmental conditions are unfavorable, vermiform males can be produced and exit roots after the 4th molt without feeding. RKN females lay their eggs outside of the body in a gelatinous matrix on the surface of the root gall. Eggs can either hatch immediately to re-infect host roots or they can overwinter in soil until the following growing season (Mitkowski and Abawi, 2003;

Karssen et al., 2013). A single life cycle can be completed in 25 days at 27°C, and multiple generations can be completed in a single growing season (Agrios, 2005).

LIFE CYCLE OF *HETERODERA GLYCINES*

Soybean cyst nematodes (SCN) (*H. glycines*) are also one of the most damaging and economically important plant-parasitic nematodes worldwide (Sasser and Freckman, 1987). The most important economic host is soybean, although SCN can infect a few minor leguminous species (Davis and Tylka, 2000). Soybean cyst nematode infection can interfere with root growth and nodulation, and cause above ground symptoms of yellowing and stunting. However, significant yield loss from SCN can occur without visible aboveground symptoms in soybean. SCN can also contribute to disease complexes with other pathogens, such as synergistic effects on sudden death syndrome in association with the fungus *Fusarium solani*. First documented in Japan in 1915, SCN is now reported in most soybean growing regions of the world, causing yield losses of 10-70% (Turner and Subbotin, 2013).

Like root-knot nematodes, soybean cyst nematodes are sedentary endoparasites (Figure 3). The life cycle takes approximately 21 days to complete at 25°C, allowing multiple generations to be completed within a single soybean growing season (Turner and Subbotin, 2013). The first molt to 2nd stage juvenile (J2) occurs inside the egg. The J2 hatches from the egg in soil and follows a chemical gradient of host root diffusate to infect host roots. J2s usually penetrate host roots behind the root tip. They then migrate intracellularly to the pericycle where secreted nematode effectors transform plant root cells into a feeding site called a syncytium. The syncytium grows and becomes multinucleate by

cell wall dissolution between adjacent root cells. Syncytium development, lack of corresponding root galls, and limited host range make cyst nematode infection of roots fundamentally different than infection by RKN. As the nematodes feed, they become sedentary and their bodies swell as they progress through a series of molts. Upon the 4th molt, lemon-shaped females of *H. glycines* rupture the root cortex, exposing their posterior end, and males, now vermiform and free-living, exit roots to fertilize exposed females. Up to a third of the eggs will be laid in an egg sac secreted by the ovaries, while a majority of eggs are retained inside the female body. The egg-laden female corpse eventually detaches from the root and undergoes a cuticle tanning process, forming a protective cyst that encases the eggs and can serve as a survival structure for years until a susceptible host is planted (Davis and Tylka, 2000; Turner and Subbotin, 2013).

NEMATODE MANAGEMENT

Once plant-parasitic nematodes become established in a field, it is nearly impossible to eradicate them. Therefore, preventing the introduction of plant-parasitic nematodes into new areas is critical. Using certified nematode-free planting material and sanitizing equipment can help prevent the spread of nematodes to new areas (Viaene et al., 2013). However, for areas where nematodes have already been introduced, management strategies aimed at reducing initial nematode population levels at the beginning of the growing season, or reducing their rate of reproduction during the growing season can help reduce nematode population levels below the threshold where economic damage occurs (Vanderplank, 1963).

Genetic host resistance can be an environmentally friendly and cost-efficient means of managing nematode populations when available. A host is considered resistant if it supports little to no nematode reproduction, as opposed to susceptible hosts that support substantial amounts of nematode reproduction. There can be varying levels of resistance that can be controlled by one or multiple genes. For example, many tomato varieties carry *Mi-1.2*, a single dominant gene known to confer resistance to multiple species of *Meloidogyne*, including *M. incognita*, *M. arenaria*, and *M. javanica*, by inducing a hypersensitive response (HR) that prevents the formation of nematode feeding sites (Williamson, 1998; Fuller et al., 2008). Similarly, the *Rk* gene in tobacco is also known to provide resistance to *M. incognita* race 1 and 3 by inducing a HR (Yi et al., 1998). In contrast, resistance to the soybean cyst nematode is often under the control of multiple quantitative trait loci (QTL) that can be dominantly inherited, such as *Rhg4*, or recessively inherited, such as *rhg1*. The source of such QTLs is often plant introductions (PI), with breeders traditionally relying heavily on PI88788 and Peking for SCN resistance (Concibido et al., 2004). Though host resistance can be very effective, resistance can be broken if resistant cultivars are planted sequentially. Continuous planting of the same resistant cultivars places high selective pressure on the nematode population that can lead to an increase in the incidence of virulence within the nematode population and eventually a loss of efficacy of the resistant crop genotype. Specific efforts, such as rotating to other sources of resistance or non-host crops, must be made to reduce the selection pressure for virulence in the nematode population and lengthen the durability of resistance. Resistance can also be associated with undesirable economic traits

such as low yield or quality, a characteristic known as linkage-drag, and it can take several years to breed new commercially acceptable resistant cultivars once a source of resistance is broken (Cook and Evans, 1987; Starr et al., 2013).

Rotation to non-host, poor-host, or antagonistic crops is often practiced so that nematode population densities will fall below the economic threshold such that a susceptible host can be profitably grown. For example, when soybeans are rotated with a non-host crop, such as corn, soybean cyst nematode populations decline as a portion of eggs hatch spontaneously and subsequently die due to the absence of a susceptible host. Poor hosts, such as resistant soybean cultivars that support little nematode reproduction can also be incorporated into a rotation scheme, allowing multiple years of a profitable soybean crop prior to rotation to corn or another non-host crop. Antagonistic crops that have toxic effects on nematodes can also be used to reduce soil nematode population levels. For example, marigolds (*Tagetes* spp.) have been shown to reduce populations of *Pratylenchus* spp. and *Meloidogyne* spp. due to a toxic compound, alphaterthienyl, contained in the roots (LaMondia, 2006; Viaene et al., 2013).

While crop rotation can be helpful in reducing soil nematode populations, like all control methods, it has its limitations. The ability of some nematodes, such as *Meloidogyne* species, to infect a wide range of host plant species, as well as the presence of multiple nematode species in a single field, may limit options for rotation crops, and rotational crops that are available may not be as profitable as the primary economic crop. For example, even though *Tagetes* species have been shown to have antagonistic effects on nematodes, they

have little commercial value (LaMondia, 2006). Furthermore, efficacy of crop rotation is dependent on length of rotation. For nematode species that can survive for long periods of time in soil, such as *Heterodera* and *Globodera* species, rotation periods of several years may be required for effective nematode management. However such rotation schemes may not be practical for farmers who grow specialized crops, have limited land, or cannot make a profit off of rotation crops. Alternatively, antagonistic crops can be grown as cover crops during the off-season; however, if this period coincides with a natural period of nematode dormancy, results may be limited. Fallow periods with no vegetation growing on the land can also be used to essentially starve the nematode populations, but leaving the land bare makes it susceptible to erosion and, with no crop in the ground, farmers cannot make a profit, making this management strategy particularly unattractive (Viaene et al., 2013).

The use of other living organisms, biological management, has potential for use to manage plant-parasitic nematode populations (Kerry, 1987; Viaene et al., 2013). Nematode predators or parasites may directly or indirectly affect the nematodes' ability to establish or survive. Biological management is an attractive strategy for its potential as a self-sustaining system that could offer long-term nematode management. However, as living organisms that require certain environmental conditions to survive, biological control agents can sometimes be difficult to establish in a field if conditions are not right and management efficacy may be inconsistent across locations. Sometimes, rather than applying biological control organisms themselves to the soil, abiotic factors are added to soil that promote growth of naturally occurring soil organisms that are already present. The addition of organic matter or other

amendments, such as chitin, to soil can cause shifts in soil microbial communities that lead to a suppression of plant-parasitic nematode populations. However, it takes time for microbial populations to build up in soil, so often biological management does not offer a practical solution to a nematode problem.

Other methods also exist to reduce initial nematode population numbers, such as soil solarization, steaming, or flooding, but these methods are less than ideal because heat-kill methods like solarization and steaming are generally only effective against nematodes in the shallow, upper layer of soil. Drowning of nematodes through flooding, like fallow, requires that the land be out of production for an extended period of time (Maas, 1987; Viaene et al., 2013).

An effective method to quickly reduce nematode populations is the application of chemical nematicides. Chemical control of nematodes has been in practice since the late 19th century and is often used in an integrated pest management strategy with other methods of nematode control for maximum levels of control. In technical terms, a chemical is considered a nematicide if it is lethal to nematodes, whereas chemicals that alter nematode behavior are considered to be nematostats. However, the term nematicide is often used as an all-encompassing term that can refer to any chemical used to control nematodes, including both true nematicides and nematostats, and will be used as such from this point forward (Haydock et al., 2013). Nematicides have been further categorized as either fumigants or non-fumigants.

Fumigant nematicides have been in use since the introduction of carbon disulfide in the late 1800s (Hague and Gowen, 1987; Haydock et al., 2013). Following World War I, it was discovered that excess teargas, chloropicrin, could be used as a soil fumigant to control nematodes and other soil-borne pathogens. Development of other fumigants soon followed, including both halogenated hydrocarbons and methyl isothiocyanate liberators (Table 1). Halogenated hydrocarbons are thought to affect protein synthesis and respiration, whereas methyl isothiocyanate liberators inhibit respiration. Many fumigants, such as methyl bromide, chloropicrin, metam sodium, and dazomet are general biocides, killing a broad spectrum of organisms, including non-target organisms. Fumigant nematicides are incorporated into the soil where they volatilize and move throughout the soil as a gas. In some cases, their high volatility necessitates that the soil be sealed after application to prevent loss of the chemical into the atmosphere, requiring costly specialized equipment to lay down sheets of plastic (methyl bromide) or roll the soil surface with a rotating drum. Fumigant nematicides also tend to be phytotoxic, so they must be applied weeks or even months in advance of planting to prevent crop damage (Hague and Gowen, 1987; Haydock et al., 2013).

Starting in the 1960s, non-fumigant organophosphate and carbamate nematicides were developed (Table 1). Many of this initial class were insecticides or their derivatives that also demonstrated activity against nematodes. Organophosphates and carbamates are non-volatile and some are systemic in plants. Organophosphates and carbamates are much easier to apply than soil fumigants because they are usually applied to the soil as a granular

formulation or, in some cases, a liquid formulation. They also tend to be less phytotoxic than fumigants, allowing at- or post-plant applications to be made. They are essentially nerve poisons, inhibiting acetylcholinesterase (AChE), an enzyme essential for turnover of the neurotransmitter acetylcholine (ACh) (Hague and Gowen, 1987; Opperman and Chang, 1992; Haydock et al., 2013). During normal neurotransmission, the neurotransmitter, acetylcholine, is released from the pre-synaptic neuron into the synaptic cleft where it then binds to receptors on the post-synaptic nerve membrane in order to transmit the nerve impulse. Normal transmission of the signal is terminated by AChE enzymes breaking down the ACh neurotransmitters. When AChE activity is inhibited, ACh accumulates in the synapse, resulting in overstimulation of ACh receptors (Čolović et al., 2013). Nematodes are paralyzed by the overstimulation of their nerve impulses, such that they cannot find or infect a host. Without a host to feed on, the nematodes eventually deplete their internal lipid reserves and die. Relatively low concentrations of nonfumigant nematicide active ingredients are sufficient to disrupt normal nematode activity. However, since they target the nervous system, carbamates and organophosphates are also acutely toxic to mammals and many other non-target organisms that also have a nervous system (Hague and Gowen, 1987; Haydock et al., 2013). Abamectin is another non-fumigant nematicide composed of a mixture of avermectin compounds derived from a soil bacterium, *Streptomyces avermitilis*. Abamectin also targets the nervous system of invertebrates, but the mode of action is different from that of the carbamates and organophosphates so it is less toxic to mammals. Abamectin is not

ideal for a soil treatment since it dissolves poorly in water and decomposes rapidly in the soil. It has shown promising activity as a seed treatment (Monfort et al., 2006).

THE DECLINE OF AVAILABLE NEMATICIDES

A major problem for most previously employed nematicides is their inherent toxicity and potential to cause environmental damage. In 1979, DBCP, the only soil fumigant nematicide that was not phytotoxic, was banned for causing male sterility in workers manufacturing the chemical (Hague and Gowen, 1987). Only 5 years later, in 1984, all pesticides containing EDB were banned by the EPA due to its groundwater contamination and carcinogenic effects. Methyl bromide is also known to be highly toxic and has been classified as an ozone depletor. A phase-out of methyl bromide was mandated by the Montréal Protocol on Substances that Deplete the Ozone Layer in 1992 (http://ozone.unep.org/new_site/en/montreal_protocol.php). D-D mixtures have also been banned due to the high potential of the 1,2-dichloropropane component as a groundwater contaminant. The 1,3-D component has good efficacy and is currently available as Telone II, but it usually must be applied pre-plant and is relatively expensive (Hague and Gowen, 1987). An amendment to the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) in 1988 required all pesticides registered prior to 1984 to be reviewed for reregistration (<http://www.epa.gov/pesticides/regulating/index.htm>). Data on human health and ecological effects were reviewed according to current scientific and regulatory standards. Furthermore, the Food Quality Protection Act (FQPA) of 1996 amended FIFRA and the Federal Food, Drug, and Cosmetic Act (FFDCA) to require reassessment of all existing tolerances for

pesticides in food. It was determined that such tolerance reassessment would be accomplished at the same time as the reregistration process. In some cases, the EPA required registrants to provide additional data to determine whether risks were acceptable and/or make changes to the label to mitigate risks. Such requirements for extensive additional data led to the voluntary cancellation of some pesticides, including many non-fumigant organophosphate and carbamate nematicides. For example, when the EPA required additional data for characterizing risks associated with exposure of fenamiphos (Nemacur) to drinking water, the registrant, Bayer CropScience, opted to cancel registration rather than spending the significant time, manpower, and money necessary to provide additional data. In other cases, the EPA cancelled registration of products deemed to pose unacceptable risks to humans or the environment. For example, after review, Carbofuran was ineligible for reregistration due to unacceptable occupational risks to applicators, dietary risks to consumers, and ecological risks to wildlife. Many nematicides remaining on the market (Table 1) have a restricted use and may only be applied by someone who has a certified pesticide applicator's license. Bans or restrictions of a majority of nematicides and increased costs of remaining nematicides have left a dire need for new nematode management solutions (Hague and Gowen, 1987; Haydock et al., 2013).

PROPERTIES OF SPIROTETRAMAT

A promising new nematicidal candidate is spirotetramat (MoventoTM)¹. Although spirotetramat (SPT) is currently marketed by Bayer CropScience as an insecticide, it also suppresses plant-parasitic nematode populations (McKenry et al., 2009; McKenry et al., 2010). SPT is a tetramic acid derivative with many favorable properties that make it an excellent insecticide and, potentially, an effective nematicide (Fischer and Weiß, 2008).

Applied to plant foliage, SPT penetrates the leaf surface and is hydrolyzed *in planta* to an enol form (Figure 4), which is the true active form. SPT-enol has a unique mode of action in insects, inhibiting acetyl-CoA carboxylase (ACC), an enzyme essential for fatty acid biosynthesis. Spirotetramat is (Insecticide Resistance Action Committee (IRAC), Group 23) a lipid biosynthesis inhibitor (LBI), along with the tetroneic acid derivatives spirodiclofen (Envidor[®]) and spiromesifen (Oberon[®]) (Fischer and Weiß, 2008; Nauen et al., 2008). There is currently no known cross-resistance to any other existing chemical classes of insecticides, making spirotetramat a valuable rotation partner with other insecticides to prevent or manage resistance (Nauen et al., 2008).

The physicochemical properties of SPT-enol allow it to enter both the phloem and xylem transport systems of the plant, resulting in unique two-way mobility in plants (Fischer and Weiß, 2008; Vermeer and Baur, 2008). In the xylem, upward transport of water and soluble mineral nutrients from the roots to the leaves is driven by the evaporation of water

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from plant leaves and other aerial surfaces, a process known as transpiration (Taiz and Zeiger, 2010). Phloem transports photosynthates from areas where they were produced (or stored) to areas where they will be used (or stored). The flow of phloem sap is driven by the active loading of sugars into the phloem sieve tubes. This active phloem loading induces an osmotic pressure difference within the phloem such that sugars are transported from areas of high pressure (source) to areas of low pressure (sink). Thus, sugars in the phloem can be transported both upward and downward within the plant, depending on the direction of the pressure gradient (Taiz and Zeiger, 2010). Therefore, the ability of SPT-enol to enter the phloem is highly valuable as an insecticide or nematicide as it allows the active ingredient to be applied to the foliage and distributed throughout the entire plant, including the below ground roots where most plant-parasitic nematodes and some insects feed (Nauen et al., 2008; Vermeer and Baur, 2008).

In order for SPT-enol to enter the vascular system of plants, SPT must first penetrate the plant tissues and be hydrolyzed to the enol form. Addition of an adjuvant with spreading and penetrating qualities helps to maximize penetration of the leaf cuticle and entry into the underlying leaf tissues where SPT can then be converted to the active and two-way systemic enol form (Vermeer and Baur, 2008).

Fortunately, SPT has very favorable toxicological, ecotoxicological, and environmental profiles, unlike many of the older fumigant and non-fumigant nematicides (Bayer CropScience, pers. comm.). SPT and its metabolites exhibited either no or very low toxicity towards mammalian, avian, aquatic, and soil-dwelling organisms, as well as other

wildlife, such as bees, and terrestrial plants in ecotoxicological studies. Consequently spirotetramat poses no obvious unacceptable risks to ecosystems and non-target organisms when used as recommended (Maus, 2008). SPT is quickly degraded in soil, aquatic, and atmospheric systems, although it is not expected to volatilize to any significant degree. It also has low water solubility and soil mobility, resulting in a low risk for leaching into groundwater (Babczynski and Hellpointer, 2008). Furthermore, no risks to consumers have been identified due to spirotetramat residues in plant or animal products (Klempner, 2008; Sur, 2008). The low risks posed by spirotetramat allow Movento to have an unrestricted use with a signal word of “Caution”, as opposed to the more severe “Warning” or “Danger” present on many pesticide labels. Workers using the product are only required to wear the minimum amount of personal protective equipment and the restricted entry interval is 24 hours (Movento label, Bayer CropScience, 2011).

ACTIVITY OF SPIROTETRAMAT ON INSECTS

While spirotetramat poses low risk to non-target organisms, its activity ranges from good to excellent against its target class of sucking insect pests, including aphids, woolly aphids, gall aphids, root aphids, psyllids, scales, mealy bugs, and whiteflies. As a lipid biosynthesis inhibitor, SPT is particularly effective against the juvenile stages of a variety of sucking insect pests (Nauen et al., 2008). Symptoms on aphid juvenile stages include incomplete ecdysis and subsequent death. Reductions in adult female fecundity and fertility of aphids and whiteflies have also been observed. Spirotetramat efficacy was reduced when aphids were exposed via dipping versus feeding on treated leaves, suggesting that ingestion

of SPT is needed for maximum efficacy. Residual control is also excellent, with 90% aphid mortality still observed up to 11 days after treatment with SPT (Nauen et al., 2008).

ACTIVITY OF SPIROTETRAMAT ON NEMATODES

The first reports of spirotetramat having nematocidal activity came from McKenry et al. (2009; 2010). They reported varying levels of suppression of plant parasitic nematode species infecting roots of citrus, grape, and walnut. In the years following, further promising results were observed for suppression of a variety of plant parasitic nematode species infecting an array of crops, including citrus, grape, almond, walnut, potato, tomato, cotton, soybean, sugarbeet, and other fruits and vegetables (Hafez et al., 2010; Hafez and Luff, 2011a, 2011b; McKenry et al., 2011; Smiley et al., 2011; Hafez et al., 2012a, 2012b; Luff et al., 2012; Shirley et al., 2012; Sipes et al., 2013; Luff et al., 2013; Sipes, 2014; Hafez and Pudasaini, 2014a, 2014b; Bayer CropScience, pers. comm.). Spirotetramat efficacy has been the strongest and most consistent for nematode suppression on grapes, almonds, and bananas with control levels similar to the NemaCur (fenamiphos) standard, while efficacy on other perennial crops such as citrus, pineapple, and tree nuts has been moderate to good (McKenry et al., 2009; McKenry et al., 2010; McKenry et al., 2011; Sipes, 2014; Bayer CropScience, pers. comm.).

Phloem-mobility of spirotetramat-enol causes it to be distributed in the plant according to the source-sink principle, whereby phloem sap follows an osmotic pressure gradient from sources of sugar production or release (mature leaves or storage organs) to sinks where sugar is being used or stored (actively growing plant parts or storage organs)

(Vermeer and Baur, 2008). Timing applications of spirotetramat with root flush periods following flowering and again following fruit maturation, when roots (where plant parasitic nematodes feed) serve as a strong sink, has produced the best results for perennial crops (Bayer CropScience, pers. comm.). Disruption of phloem transport a few days after SPT application reduced its efficacy against nematodes, indicating that perhaps a continuous flow of SPT to nematode feeding sites in roots is required for maximum nematicidal effects (McKenry et al., 2010). Irrigating too soon after SPT application to perennial crops reduced efficacy (McKenry et al., 2009; McKenzie et al., 2011), perhaps by altering the source-sink relationship.

Variable levels of efficacy have been observed for SPT applications on annual crops, including fruiting and leafy vegetables, dry beans, soybeans, cotton, and potatoes, with the best results observed when applications are made 28-56 days after planting. Also, curative application timings have fared much better than preventative applications (Bayer CropScience, pers. comm.).

Several studies have been conducted to determine how SPT suppresses nematode populations. Knowing exactly how SPT affects nematode biology could allow fine-tuning of application timings and rates for maximum nematode control. It is suspected, though not confirmed, that SPT disrupts lipid biosynthesis in nematodes, similar to its activity in insects (Bayer CropScience, pers. comm.). Lipids serve many important roles for nematodes and disruption of lipid biosynthesis could potentially have detrimental effects. Lipids are a major source of energy storage, major components of cellular membranes, involved in signal

transduction cascades, and precursors of hormones and other biologically active molecules. A reduction of lipid reserves has been correlated with decreased infectivity in some plant-parasitic nematodes and it has been suggested that SPT may cause unsuccessful host infection due to a lack of energy (Perry et al., 2013; Bayer CropScience, pers. comm.).

In a mechanistic approach to elucidate the effects of SPT on development of southern root-knot nematodes (*Meloidogyne incognita*), roots of *M. incognita*-infected cowpea plants were stained with acid fuchsin at 1-week intervals after inoculation to track development of infecting nematodes through the juvenile stages to adulthood (Bayer CropScience, pers. comm.). A slowing of development was observed in spirotetramat-treated nematodes indicated by a higher proportion of earlier staged juveniles and a lower proportion of more mature juvenile and adult stages compared to the non-treated controls at various time points of observation. Subsequent experiments conducted with *C. elegans*, and the model *H. schachtii*-*Arabidopsis thaliana* pathosystem, revealed similar results (Bayer CropScience, pers. comm.). Effects on mixed stages of *C. elegans* were both concentration- and time-dependent. After 3 days, no effects were observed at 3ppm, developmental arrest and a lack of movement was observed on a proportion of worms at 30ppm, and no development and an increasing number of still worms was observed at concentrations ≥ 60 ppm (Bayer CropScience, pers. comm.). Furthermore, an increasing percentage of worms became still as they were incubated in 60ppm SPT for longer periods of time. *H. schachtii* females infecting *Arabidopsis* plants treated with SPT failed to molt (Bayer CropScience, pers. comm.). This suggests that the delayed development observed in *M. incognita* and *C. elegans* could be due

to an inability to molt. In addition, lipid droplet imaging of *C. elegans* exposed to SPT revealed an absence of lipid droplets under starved conditions, suggesting effects on lipogenesis (Bayer CropScience, pers. comm.).

In summary, preliminary results suggest that spirotetramat may be affecting nematodes in a similar manner to insects, with slowing/arrest of development observed in *M. incognita* and *C. elegans*, inhibition of molting observed in *H. schachtii* females, and absence of lipids under starved conditions observed in *C. elegans* (Bayer CropScience, pers. comm.). However, opportunity remains to expand on work already done and to identify further possible effects. Although suppression of juvenile development has been suggested, the relationship between SPT concentration and inhibition of juvenile development is not known. While studies have been done comparing the efficacy of different foliar application rates, it is unknown how much of the active ingredient is actually reaching nematodes in the roots and at what concentration maximum efficacy is achieved. Furthermore, little is currently known about effects of SPT on egg hatch or fecundity and fertility of females. Although there may be an effect on production of viable eggs from cyst nematodes infecting wheat plants treated with SPT, results were not statistically significant, warranting further research (Smiley et al., 2011). Studies have been conducted to compare different SPT application rates but little has been done to determine optimal application timings. Several studies have shown that curative treatments tend to work better than preventative treatments (Bayer CropScience, pers. comm.), but no known studies have tested multiple application timings during the first life cycle of infecting nematodes to determine the best time to apply SPT in order to target

susceptible nematode life stages for optimal nematode control. Field trials testing efficacy of SPT against *H. avenae* infecting wheat suggested differences in the magnitude of reduction of *H. avenae* density in soil at different field locations could be due to different application timings corresponding to developmental stages of infecting nematodes. Further research is warranted to determine optimal SPT application timings to nematode developmental stages (Smiley et al., 2011).

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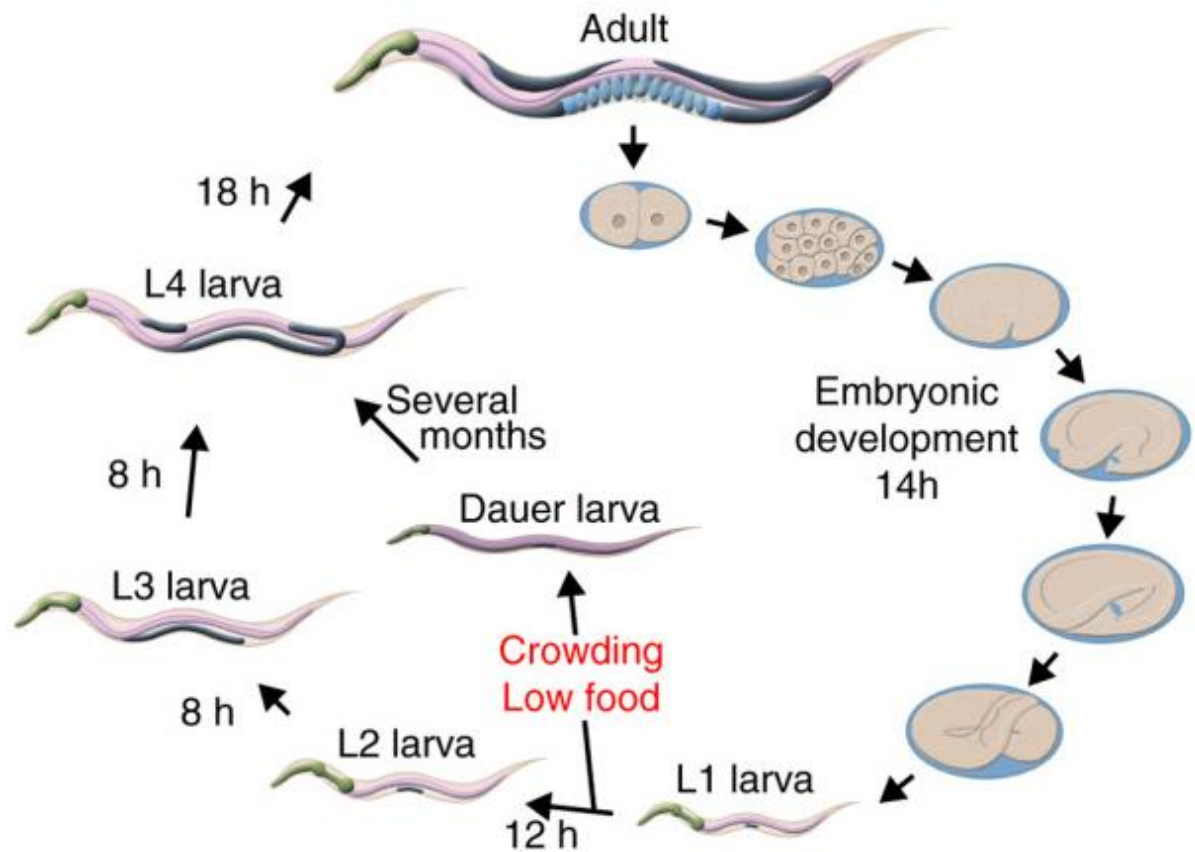


Figure 1. Life cycle of *Caenorhabditis elegans*. After undergoing embryonic development inside the egg, the first (L1) larval stage hatches and progresses through 4 molts before becoming an adult hermaphrodite (a small minority can become males). Under sub-optimal conditions, remaining larval stages develop into an alternate third larval survival stage, called a dauer, which is capable of surviving in an arrested inactive state for several months until growth conditions become favorable, at which point normal development resumes (Reproduced from Murgatroyd and Spengler, 2010).

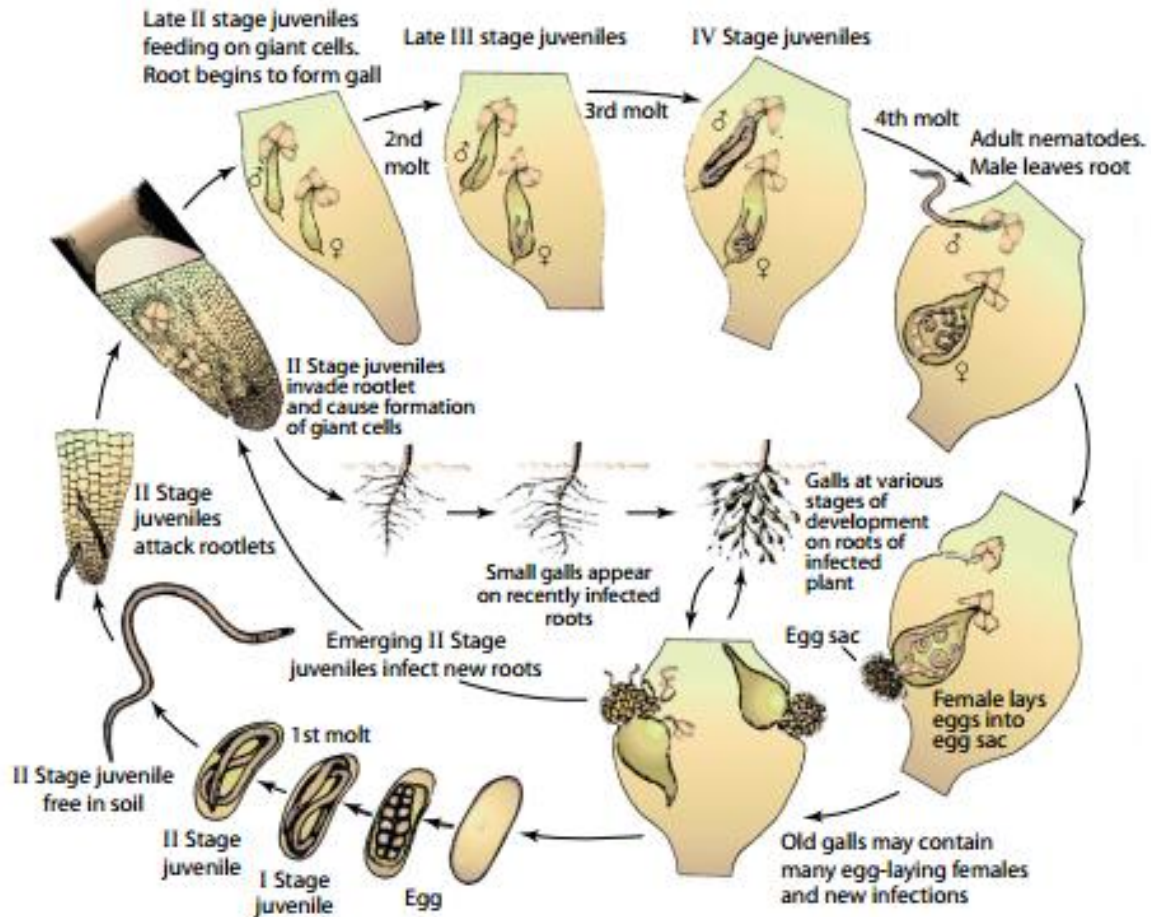


Figure 2. Life cycle of root-knot nematode (*Meloidogyne* spp.). The first molt to second-stage juvenile (J2) occurs inside the egg. After hatching, the infective J2 penetrates a host root behind the tip and induces a permanent feeding site (giant-cells) from selected root vascular cells. A localized gall (knot) of root tissue forms surrounding the infection site. As the nematodes progress through a series of molts, the females become swollen and flask-shaped, while a few (non-sexual) vermiform males exit the root after the 4th molt. The female lays eggs (usually formed by parthenogenesis) in a gelatinous matrix outside the female body on the surface of the gall (Reproduced from Agrios, 2005).

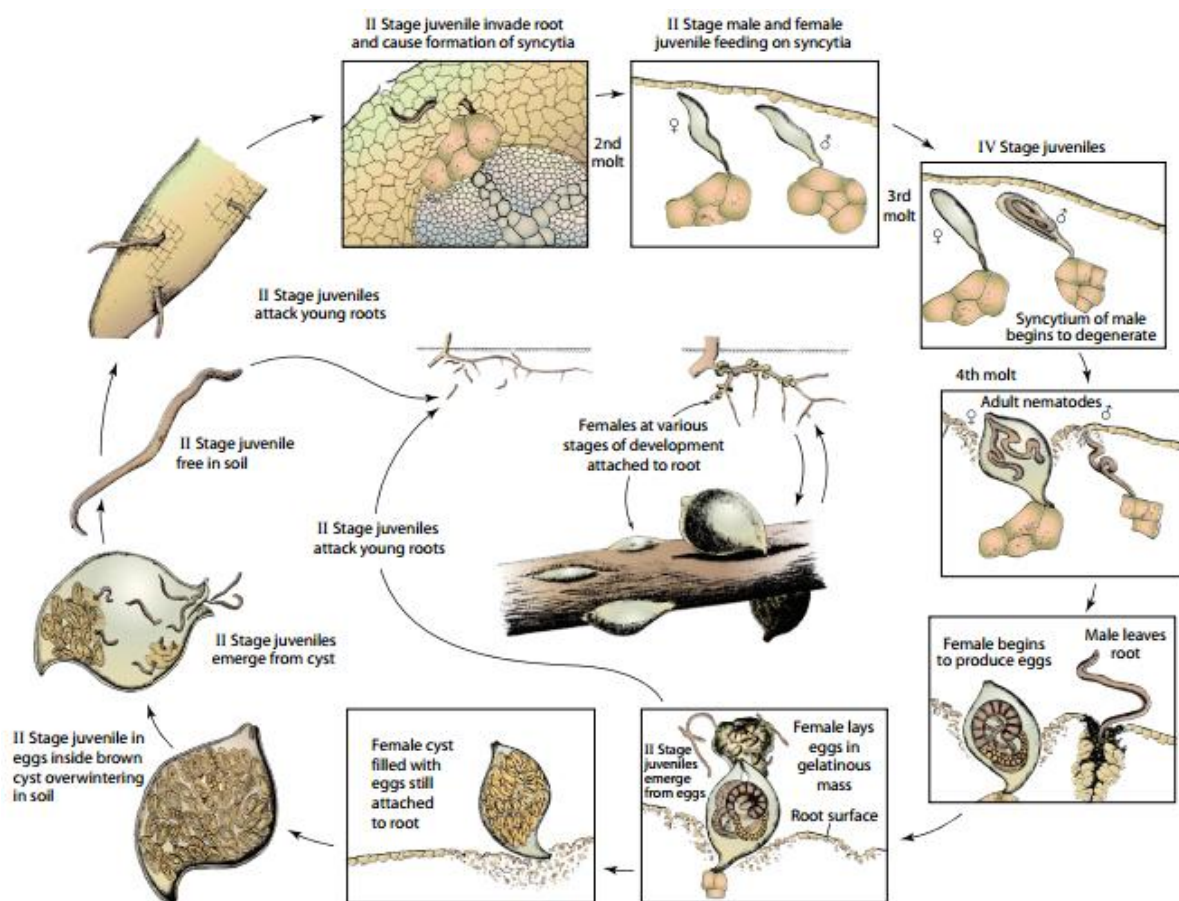


Figure 3. Life cycle of soybean cyst nematode (*Heterodera glycines*). The first molt occurs inside the egg. The infective J2s hatch and emerge from the protective cyst to seek out a host. After penetrating the host roots, the J2s induce a feeding site in the root vasculature, called a syncytium, and progress through a series of molts as they feed. As the female bodies swell, they rupture the root (no gall is formed), exposing their posterior end. The vermiform males leave the roots to fertilize the females. A portion of eggs are laid in an egg sac, while a majority of eggs are retained in the female body which will fall off the root and form a protective cyst in the soil (Reproduced from Agrios, 2005).

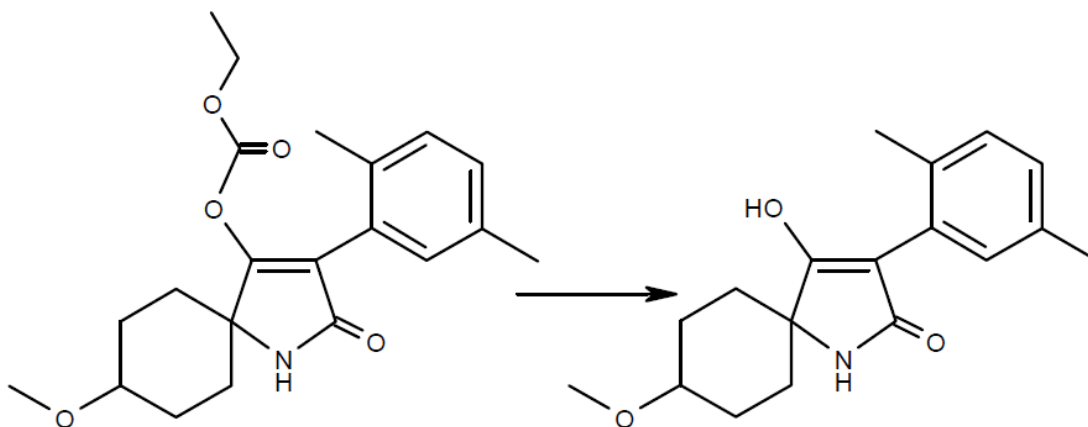


Figure 4. *In planta* hydrolyzation of spirotetramat. Spirotetramat (left) is an insecticide marketed as Movento™ (Bayer CropScience) with two-way systemic activity and is applied to plant foliage. Once spirotetramat penetrates the leaf cuticle, it is hydrolyzed to the active enol form (right) *in planta*. The enol form is ambimobile and functions as a lipid biosynthesis inhibitor (Reproduced from Bayer CropScience).

Table 1. Historically-important nematocides.

Active Substance	Chemical Group	Mode of Application	Example Trade Name	Currently Available
Chloropicrin	Halogenated hydrocarbon	Fumigant	Chloropicrin 100 Fumigant	Yes*
1,3-dichloropropene (1,3-D)	Halogenated hydrocarbon	Fumigant	Telone II	Yes*
Methyl bromide	Halogenated hydrocarbon	Fumigant	MeBr	No
1,2-dibromo-3-chloropropane (DBCP)	Halogenated hydrocarbon	Fumigant	Nemagon	No
1,3-dichloropropene – 1,2-dichloropropane mixtures (D-D)	Halogenated hydrocarbon	Fumigant	Vorlex	No
Ethylene dibromide (EDB)	Halogenated hydrocarbon	Fumigant	Dowfume	No
Metam sodium	Isothiocyanate liberator	Fumigant	Vapam	Yes*
Dazomet	Isothiocyanate liberator	Fumigant	Basamid	Yes*
Fenamiphos	Organophosphate	Non-fumigant	Nemacur	No
Ethoprophos	Organophosphate	Non-fumigant	Mocap EC	Yes*
Thionazin	Organophosphate	Non-fumigant	Nemafos	No
Cadusafos	Organophosphate	Non-fumigant	Rugby	No
Fosthiazate	Organophosphate	Non-fumigant	Nemathorin	Yes
Carbofuran	Carbamate	Non-fumigant	Furadan	No
Aldicarb	Carbamate	Non-fumigant	Meymik	Yes*
Oxamyl	Carbamate	Non-fumigant	Vydate	Yes*
Abamectin	Avermectins	Non-fumigant	Avicta	Yes*

*Restricted Use

SPIROTETRAMAT-ENOL CAUSES AN ARREST OF JUVENILE DEVELOPMENT IN NEMATODES

ABSTRACT

The insecticide spirotetramat (Movento™) also suppresses plant nematode populations. Inside the plant, spirotetramat is hydrolyzed to the active enol form that functions as a lipid biosynthesis inhibitor. Little is known about effects of spirotetramat (SPT) on nematode biology. The objective of this research was to determine what nematode life cycle stages were most affected by SPT-enol. Hatching tests were conducted with the model nematode *Caenorhabditis elegans*, with no significant ($p < 0.05$) effects on hatching rates observed at a maximum SPT-enol concentration of 105ppm. SPT-enol also did not affect hatching of plant parasitic *Meloidogyne incognita* (southern root-knot nematode) and *Heterodera glycines* (soybean cyst nematode). Life-stage assays conducted with *C. elegans* indicated spirotetramat-enol concentrations as low as 30ppm ($p < 0.05$) arrested juvenile development before reaching adulthood. Dose response curves generated from life-stage experiments estimated SPT-enol EC_{95} to be between 44 and 48ppm.

INTRODUCTION

Root-knot nematodes (*Meloidogyne* spp.) cause the most economic crop damage of any nematode group, infecting more than 2,000 species of plants worldwide (Sasser and Freckman, 1987; Mitkowski and Abawi, 2003). Cyst nematodes (*Heterodera* and *Globodera* spp.), are second in agricultural importance - the soybean cyst nematode (*Heterodera*

glycines) is the leading pathogen of soybean worldwide (Sasser and Freckman, 1987; Davis and Tylka, 2000).

Many different strategies have been employed to manage nematode damage, including planting of resistant cultivars, rotating with non- or poor-host crops, as well as biological control (Cook and Evans, 1987; Kerry, 1987; Starr et al., 2013; Viaene et al., 2013). However, the cost-efficiency and durability of these management strategies is dependent on multiple factors including the crop host, species and population levels of the nematodes present, nature and availability of genetic resistance in the crop species, and a plethora of environmental factors.

Chemical control (soil fumigants and nonfumigant nematicides) can offer an effective means of management of plant-parasitic nematodes that is independent of the nematode species present and many environmental conditions. Chemical control of nematodes has been in practice since the late 19th century and often used in combination with other nematode control methods in an integrated pest management strategy (Cook and Evans, 1987; Kerry, 1987; Starr et al., 2013; Viaene et al., 2013). However, the number of commercially available chemical nematicides has declined in recent years due to their inherent toxicity and potential to cause environmental damage. Since the late 1970's, bans and restrictions of a majority of nematicides have left a dire need for new nematode management solutions (Hague and Gowen, 1987; Haydock et al., 2013).

Spirotetramat (MoventoTM, Bayer CropScience), a tetramic acid derivative, is currently labeled as an insecticide but has also been demonstrated to suppress nematode

populations (McKenry et al., 2009; McKenry et al., 2010). Spirotetramat (SPT) has a unique insecticidal mode of action (IRAC, Group 23) as a lipid biosynthesis inhibitor that reduces lipid content, inhibits ecdysis, and reduces fecundity and fertility (Nauen et al., 2008). Spirotetramat also has very favorable toxicological, ecotoxicological, and environmental profiles (Babczynski and Hellpointer, 2008; Klempner, 2008; Maus, 2008; Sur, 2008).

Applied to plant foliage, SPT penetrates the leaf surface and is hydrolyzed *in planta* to the active enol form. The physicochemical properties of SPT-enol allow it to enter both phloem and xylem transport systems of the plant, resulting in unique two-way systemicity that allows the active ingredient to travel from the point of application into the foliage and down into the roots where nematodes and some insects feed (Fischer and Weiß, 2008; Vermeer and Baur, 2008).

McKenry et al. (2009) first reported varying levels of suppression by SPT of plant-parasitic nematode species infecting citrus, grape, and walnut. Subsequent field studies have been done to determine which crops and nematode populations are suppressed by SPT (Hafez et al., 2010; Hafez and Luff, 2011a, 2011b; McKenry et al., 2011; Smiley et al., 2011; Hafez et al., 2012a, 2012b; Luff et al., 2012; Shirley et al., 2012; Sipes et al., 2013; Luff et al., 2013; Sipes, 2014; Hafez and Pudasaini, 2014a, 2014b; Bayer CropScience, pers. comm.). However, exactly how SPT affects nematode biology remains unknown – results that could increase the utility and effectiveness of SPT to reduce crop damage from plant-parasitic nematodes. Preliminary results suggest that SPT may affect nematodes in a similar manner to insects, with slowed/arrested development observed in *M. incognita* and *C.*

elegans, inhibition of molting observed in *H. schachtii* females, and absence of lipids under starved conditions observed in *C. elegans* (Bayer CropScience, pers. comm.).

Although an effect on nematode development has been suggested, the relationship between SPT concentration and inhibition of nematode development is not known. While many studies have been conducted comparing the efficacy of different foliar application rates, it is unknown how much of the active ingredient is actually reaching the nematodes in the roots and at what concentration maximum efficacy is achieved. Furthermore, little is currently known about the effects of SPT on egg hatch or fecundity and fertility of females. Fully understanding the effects of SPT-enol on nematode biology could allow foliar SPT applications to be timed according to when nematodes are in the most vulnerable stages for optimal nematode control. Furthermore, knowing what concentration of SPT-enol is needed for maximum efficacy could help pave the way for more efficient usage of SPT such that the amount of SPT applied is adequate for effective nematode control without being excessive. Thus, with proper timing and rate driven by effects on nematode biology, the efficiency of SPT applications could be optimized to offer maximum nematode control with minimum cost and environmental impact.

The objective of this study was to determine the nematode life cycle stages most affected by SPT-enol. Hatching and staged assays with the model nematode, *Caenorhabditis elegans* were conducted because, unlike sedentary endoparasitic root-knot and soybean cyst nematodes which spend a majority of their month-long life cycles embedded in host roots, *C. elegans* has a short life cycle that can be completed in a petri dish and is easily observable in

laboratory studies (Jones et al., 2011). A dose-response curve was developed to model the relationship between SPT-enol concentration and observed effects on development of *C. elegans*. Hatching tests were repeated with plant-parasitic nematodes, *Meloidogyne incognita* and *Heterodera glycines*, in order to show that results obtained with *C. elegans* could be extended to economically important species of plant-parasitic nematodes.

MATERIALS AND METHODS

C. elegans culture: *C. elegans* wild-type strain, N2, stock cultures were maintained on NGM plates seeded with the OP50 strain of *Escherichia coli* as described previously (Brenner, 1974). Large quantities of gravid *C. elegans* hermaphrodites were grown on 15-cm peptone-enriched plates of media seeded with the NA22 strain of *E. coli* (Schachat et al., 1978; Lewis and Fleming, 1995). Eggs were harvested from these gravid adults by treatment with a hypochlorite solution (recipe 5) for 4 minutes as previously described (Porta-de-la-Riva et al., 2012).

H. glycines and *M. incognita* culture: *H. glycines* were propagated on roots of soybean plants (*Glycine max* cv Hutcheson) grown in the greenhouse. Cysts were dislodged from soybean roots with a directed water stream and collected in a 250 μ m sieve. Cysts were then crushed with a rubber stopper over a 25 μ m sieve to collect eggs (Goellner et al., 2001). *Meloidogyne incognita* were propagated on roots of tomatoes (*Solanum lycopersicum* cv Rutgers) grown in the greenhouse. *M. incognita* eggs were extracted from host roots by rinsing roots in 0.6% sodium hypochlorite for 30 seconds as previously described (Hussey

and Barker, 1973) and collecting and rinsing the eggs on a 25 μ m mesh sieve. Both *H. glycines* and *M. incognita* eggs were cleaned via the sugar flotation method (Agrios, 2005).

Hatching assays with C. elegans: Hatching assays were first conducted with the model nematode, *C. elegans* to determine the effect of SPT-enol on egg hatch rate. *C. elegans* hatching assays were conducted at room temperature in 96-well plates, with a single well equaling one replication. Approximately 100 eggs in M9 buffer were pipetted into each well and then SPT-enol (dissolved in either 50% acetone-triton [5-mL Triton X-100 per L of Acetone, Test 1] or 20% dimethyl sulfoxide [DMSO, Test 2]) was added to a final concentration of either 105ppm, 90ppm, 75ppm, 60ppm, 45ppm, 30ppm, or 3ppm, bringing the total volume to 100- μ L. Buffer and solvent controls were included, as well as a positive nematicidal control of 0.1ppm abamectin (ABM), for a total of 10 treatments. Each treatment was replicated five times. The number of first-stage larvae (L1s) of *C. elegans* that hatched in each well after 3 days was counted and percent hatch calculated.

Hatching assays with H. glycines and M. incognita: To see if results from hatching assays conducted with *C. elegans* were comparable to economically important plant-parasitic nematode species, hatching tests were also conducted with *H. glycines* and *M. incognita*. *H. glycines* and *M. incognita* eggs were hatched at 28°C in bowls using the Baermann technique. Approximately 20,000 eggs were placed on a double-layered Kimwipe™ on a mesh screen placed inside a plastic bowl. SPT-enol (dissolved in 20% DMSO) was used to prepare hatching solutions at concentrations of 90ppm, 60ppm, and 30ppm. Water and solvent controls were also included for a total of 5 treatments. Hatching solutions were

poured into respective Baermann bowls such that the surface of the liquid enveloped the eggs hatching through the screen. Five replications were included per treatment. Hatched second-stage juveniles (J2s) of *H. glycines* and *M. incognita* were collected from the bowls for 7 days and combined. Hatched J2s were then counted and percent hatch was calculated.

Staged developmental assays with C. elegans: To determine the effects of SPT-enol on nematode development, synchronized *C. elegans* populations starting with hatched first-stage larvae (L1) were observed over the course of one life cycle. *C. elegans* populations were synchronized by plating eggs onto water agar plates with no food and collecting the hatched L1s in M9 buffer after 24-48 hours (Lewis and Fleming, 1995). Approximately 40 L1s were pipetted into each well of 24-well plates along with 800- μ L of test medium (10-mL of OP50 *E. coli* overnight culture, 5-mg of ampicillin, and 2-mg of nystatin per 100-mL of M9 buffer). SPT-enol was then added to a final concentration of either 105ppm, 90ppm, 75ppm, 60ppm, 45ppm, 30ppm, or 3ppm, bringing the total volume to 1-mL/well. Buffer and solvent controls were included, as well as positive nematicidal controls of 0.1ppm ABM and 10ppm mebendazole (MBZ), for a total of 11 treatments. ABM is a mixture of avermectin compounds derived from the soil bacterium, *Streptomyces avermitilis*, and works by targeting the nervous system. Nematodes become paralyzed by low doses and, thus, ABM was used as a comparison for acute toxicity (Martin et al., 2002). MBZ is an antihelmintic drug that inhibits the synthesis of microtubules in the intestine, blocking uptake of nutrients, causing a more gradual paralysis than ABM (Spence et al., 1982). Therefore, MBZ was used as a comparison for chronic effects on nematode development. Five replicates were included

for each treatment. Developmental progress of treated staged *C. elegans* was observed over the course of 4 days and then the number of adult worms in each well was recorded.

Statistical analysis: Data were log transformed prior to analysis to satisfy assumptions of normality and homogeneity of variance ($\log(y)$ for *C. elegans* percent hatch, $\log(y+10)$ for *H. glycines* and *M. incognita* percent hatch, and $\log(y+1)$ for counts of developed *C. elegans*). A two-way ANOVA was performed for duplicate experiments using the general linear model (GLM) procedure in SAS version 9.4 (SAS Institute, Cary, NC) to check for experiment by treatment interactions. If interaction effects were not significant then data were combined across experiments for subsequent analysis. If interaction effects were significant ($p < 0.05$) then further analysis was conducted on individual experiments. When treatment means were significant by ANOVA, means were separated using the Scheffé test ($\alpha = 0.05$).

Dose response model: The relationship between SPT-enol concentration and the number of *C. elegans* L1s that fully developed to adults was modeled for each independent staged assay by fitting original data to a three-parameter log-logistic regression model (Eqn. 1) using the statistical software R version 3.0.3 (<http://www.r-project.org/>) with the analysis of dose response curves (drc) package (Ritz and Streibig, 2005).

$$y = \frac{d}{1 + \exp[b\{\log x - \log e\}]} \quad (1)$$

In the model, x = the SPT-enol concentration in ppm, while the parameters e , b , and d represent the SPT-enol concentration in which the number of developed juveniles is reduced by half (EC_{50}), the slope around e , and the upper-response limit of the curve.

RESULTS

Hatching assays with C. elegans: No effects on *C. elegans* hatch rate were observed (Figure 5A) even at the maximum SPT-enol concentration tested (105ppm). Although two-way ANOVA indicated significant ($p < 0.05$) experiment by treatment interactions, the same conclusions were reached when individual analysis of each independent assay was conducted, with no significant differences between the SPT-enol treatments and buffer and solvent controls (Scheffé test, $\alpha = 0.05$).

Hatching assays with H. glycines and M. incognita: Two-way ANOVA did not indicate any significant experiment by treatment interactions, so the data from independent assays were combined for further analysis. There were no significant effects on *H. glycines* or *M. incognita* hatch rate caused by SPT-enol observed even at the maximum concentration tested, 90ppm (Figure 5B and 5C, respectively). These results were similar to those obtained with *C. elegans*. *H. glycines* did appear to be sensitive to the DMSO solvent, as indicated by the significant reduction in hatch rate in the DMSO control treatment and all SPT-enol treatments compared to the water control. However, no significant differences in *H. glycines* hatch between the solvent control and any SPT-enol treatment were observed.

Staged developmental assays with C. elegans: No acute (lethal) effects were observed in worms soaked in SPT-enol of any concentration. Worms soaked in 0.1ppm ABM became

immobile within 24 hours, whereas many worms soaked in the highest concentration of SPT-enol (105ppm) continued to move normally for days after initial exposure. Some worms in higher concentrations of SPT, however, did appear to have labored movement after 2-3 days, similar to those treated with 10ppm MBZ. Although *C. elegans* soaked in SPT-enol were not killed immediately and continued to move for days, they remained small, suggesting an arrest of juvenile development. Juveniles in SPT-enol treatments that had not fully developed to adults after one life cycle (3-4 days) continued to be observed for up to 10 days (the equivalent of at least three life cycles), but complete development to adult was never observed. A significant ($p < 0.05$) reduction in the number of juveniles that fully developed was observed in SPT-enol concentrations ≥ 30 ppm (Figure 6). Although two-way ANOVA indicated significant ($p < 0.05$) experiment by treatment interactions, the same conclusions were reached when individual analysis of each independent assay were conducted. In each assay, the number of juveniles that failed to develop to adults was dependent on the concentration of SPT-enol. Dose response curves generated for each independent staged *C. elegans* developmental assay estimated an EC_{95} between 48 and 44ppm (Figure 7).

DISCUSSION

A delay in nematode juvenile development caused by SPT was observed in previous research (Bayer CropScience, pers. comm.). Cowpeas were sprayed with Movento at rates equivalent to 70.08 g ai/ha (4-fl oz/acre) and 109.5 g ai/ha (6.25-fl oz/acre) at 2 days after inoculation with 2000 *M. incognita* J2s. Infected roots were then stained and dissected in 1-week intervals following inoculation with nematodes. A significant increase in the proportion

of J3s and J4s coinciding with a significant decrease in the proportion of females as compared to the non-treated controls was observed at 20 days after inoculation, suggesting a delay in juvenile development caused by SPT treatment. Studies (Bayer CropScience, pers. comm.) with mixed life stages of *C. elegans* suggested that SPT effected *C. elegans* development and movement at concentrations ≥ 30 ppm. The relationship between SPT concentration and observed effects on nematode development could not be accurately modeled using mixed life stages. In the present study, previous research was expanded by conducting hatching tests with *C. elegans*, *H. glycines*, and *M. incognita* as well as generating a concentration response curve based on data from staged assays with *C. elegans*.

SPT-enol had no apparent effect on the hatching rate of *C. elegans*, *H. glycines*, and *M. incognita* eggs soaked in SPT-enol concentrations as high as 105ppm. It is possible that SPT-enol truly had no direct effect on ability to hatch or that the eggshells were simply impervious to the chemical. Either way, for practical purposes direct application of SPT-enol does not seem to have an effect on egg hatch of multiple nematode species. These tests were only conducted with first generation eggs that had been collected from stock plants that were never treated with SPT. No direct evidence has been generated to test if SPT-enol has an effect on the hatch rate or viability of second generation eggs produced by females feeding on plants treated with SPT. Further research to study potential chronic effects on fertility or fecundity of females exposed to SPT-enol within treated host plants is warranted. Evaluation of female nematode fecundity after plant host treatment with SPT and observation of reproductive rates over at least two nematode generations could provide evidence about the

effects of SPT on proper egg formation and viability of a second generation of nematodes (i.e. infectivity of hatched J2 from SPT-treated plants).

Results of staged assays with *C. elegans* were consistent with those from previous research with mixed life stages, indicating an arrest of juvenile development before reaching adulthood. Significant reductions in the number of worms that developed to adults were observed in SPT-enol concentrations ≥ 30 ppm. While previous studies with *M. incognita* had suggested a delay in juvenile development, results from the present study suggest an arrest, rather than a delay, as juveniles in SPT-enol treatments that had failed to fully develop by 3 or 4 days (normal life cycle), still did not develop fully when observation of assays was extended to 10 days (the equivalent of 3 or more life cycles). While it seems that SPT-enol causes an arrest of juvenile development, it is still unknown whether these effects are reversible. Nematodes exposed to a range of SPT-enol concentrations for varying lengths of time and then transferred to media free of SPT-enol could be investigated to see if normal development resumes.

The number of juveniles that failed to develop in SPT-enol treatments was dependent on SPT-enol concentration. Dose response models generated for each independent assay indicated the EC₉₅ to be 44 and 48ppm, respectively. Nematode juveniles appear to be somewhat less sensitive to SPT-enol than insects, as leaf-dip bioassays with SPT revealed the LC₉₅ for aphid, *Aphis gossypii*, nymphs to be 21ppm (Nauen et al., 2008). Adult aphid fecundity and fertility were also lower, observed at SPT concentrations down to 1ppm. Whiteflies, *Bemisia tabaci*, are also highly sensitive, with the LD₅₀ for nymphs below 1ppm.

Adult whitefly fecundity and fertility are also affected, with 90% reductions in fecundity observed at 200ppm and 60% reductions observed at 40ppm. However, at 40ppm, almost all eggs failed to hatch and even at 8ppm, 80 % of the eggs still failed to hatch (Nauen et al., 2008). Even though EC₉₅ values for nematodes are not as low as those for sucking insects, they are still well below the labeled insecticidal rate of 936ppm (87.6 g ai/ha or 5 fl oz/acre). Efficacy against nematodes infecting plant roots has been observed when the insecticidal rate was applied to plant foliage, suggesting that sufficient amounts of SPT are getting converted to the active enol form and translocated to the roots where nematodes feed (Bayer CropScience, pers. comm.).

SPT-enol is known to be a lipid biosynthesis inhibitor (LBI). Nematodes have many lipid stores which they use as a source of energy (Perry et al., 2013). As nematodes deplete their energy reserves and are unable to restore them by synthesizing new fatty acids, a lack of energy needed for development could explain the developmental arrest caused by SPT-enol. In a field setting, this lack of development would likely manifest as a gradual decrease in the population due to a reduction in reproduction rate. Effects on nematode development are similar to effects observed in insects. SPT is known to be particularly effective against juvenile stages of a variety of sucking insect pests, with symptoms on aphids including incomplete ecdysis and subsequent death (Nauen et al., 2008). Since incomplete ecdysis has been observed in insects, and, like insects, nematodes are also invertebrates that molt their exoskeletons during development, the results of these and related investigations suggest that SPT-enol might inhibit molting and thus, normal development in nematodes. The outermost

layer of the nematode cuticle, the epicuticle, is a trilaminar layer composed of proteins and lipids. This is the first layer of the cuticle to be laid down during molting (Decraemer and Hunt, 2013). Perhaps a shortage of lipids needed to construct the new cuticle during molting is responsible for an inhibition of molting and an arrest of juvenile development. Further research could study the direct effects of SPT-enol on nematode molting and lipid synthesis in worms.

In the present study, staged assays were conducted *in vitro* with the free-living nematode, *C. elegans*. The natural next step would be to conduct *in planta* infection assays in the greenhouse with economically important plant-parasitic nematodes to see if results are comparable. Knowing that SPT-enol inhibits juvenile development, perhaps foliar SPT applications could be timed according to when nematodes are in vulnerable juvenile stages for maximum control. Field studies should also be conducted to see how application timings targeted at juvenile stages translate into a field setting where nematode stages are asynchronous. Perhaps the best timing would be at the beginning of the growing season, shortly after planting, when nematodes are beginning to hatch and infect. Nonetheless, not all eggs would hatch immediately and some would likely escape exposure if the residual activity of SPT-enol was too short. Further testing could be done to determine the residual effect of a single application. If residual effects do not last long enough to adequately expose multiple generations, multiple applications may be necessary. Strategic timing of applications during vulnerable nematode stages or root flush periods that permit maximum delivery of SPT-enol

to nematode feeding sites in roots could optimize SPT efficiency and maximize nematode control.

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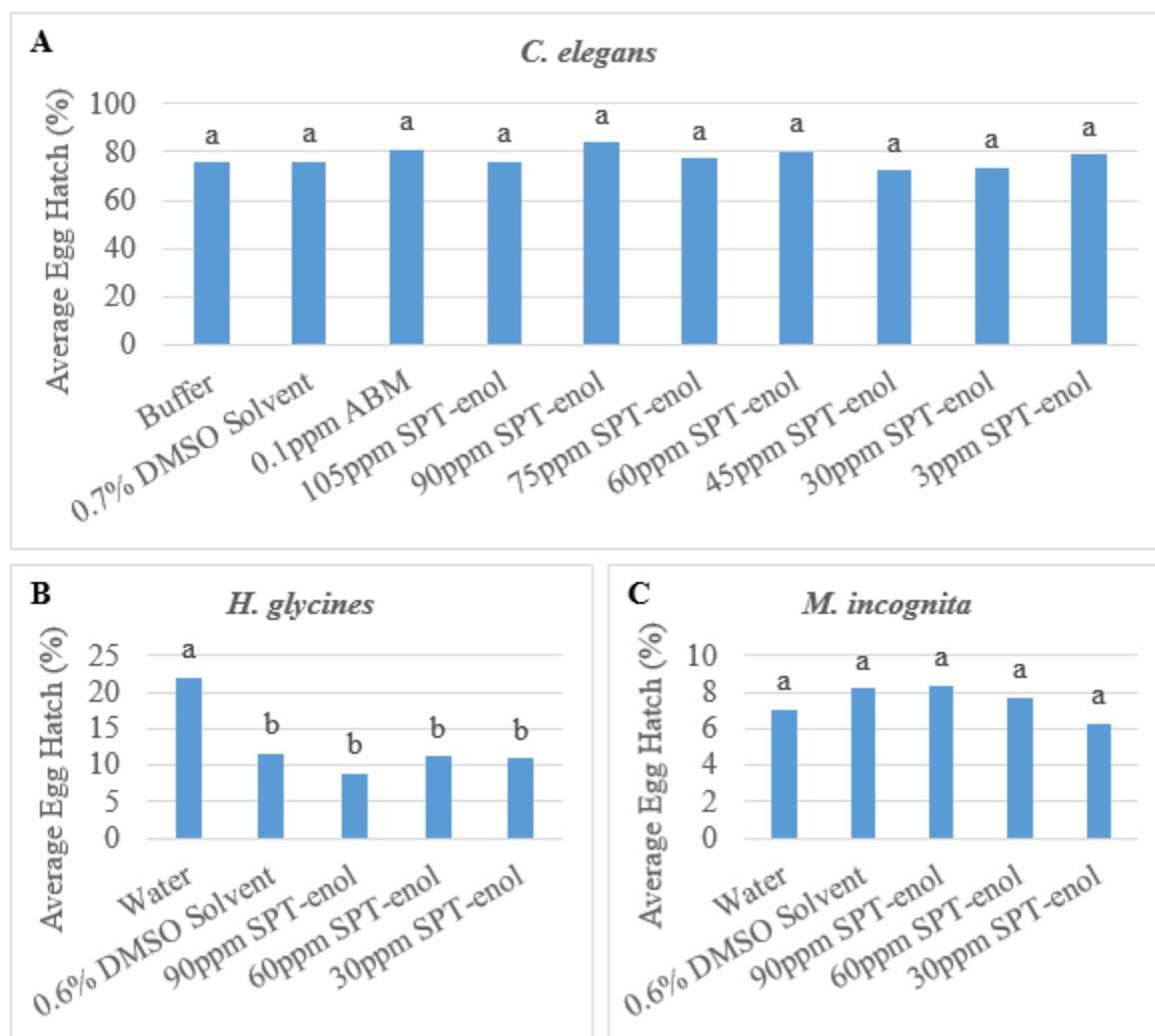


Figure 5. Percent hatch for *C. elegans* (A), *H. glycines* (B), and *M. incognita* (C) eggs immersed in a range of spirotetramat-enol (SPT-enol) concentrations. No significant reductions in percent egg hatch caused by SPT-enol were observed for any nematode species by two-way ANOVA ($p < 0.05$), although, *H. glycines* did appear to be sensitive to the DMSO solvent. Differing letters represent a statistical difference (Scheffé test, $p < 0.05$). Data was transformed ($\log(y)$ for *C. elegans* and $\log(y+10)$ for *H. glycines* and *M. incognita*) prior to statistical analysis; however, non-transformed data are presented in the graphs for ease of interpretation. Data in A are from a single representative hatching test with *C. elegans*. Data in B represent an average across two *H. glycines* hatching tests. Data in C represent an average across two *M. incognita* hatching tests.

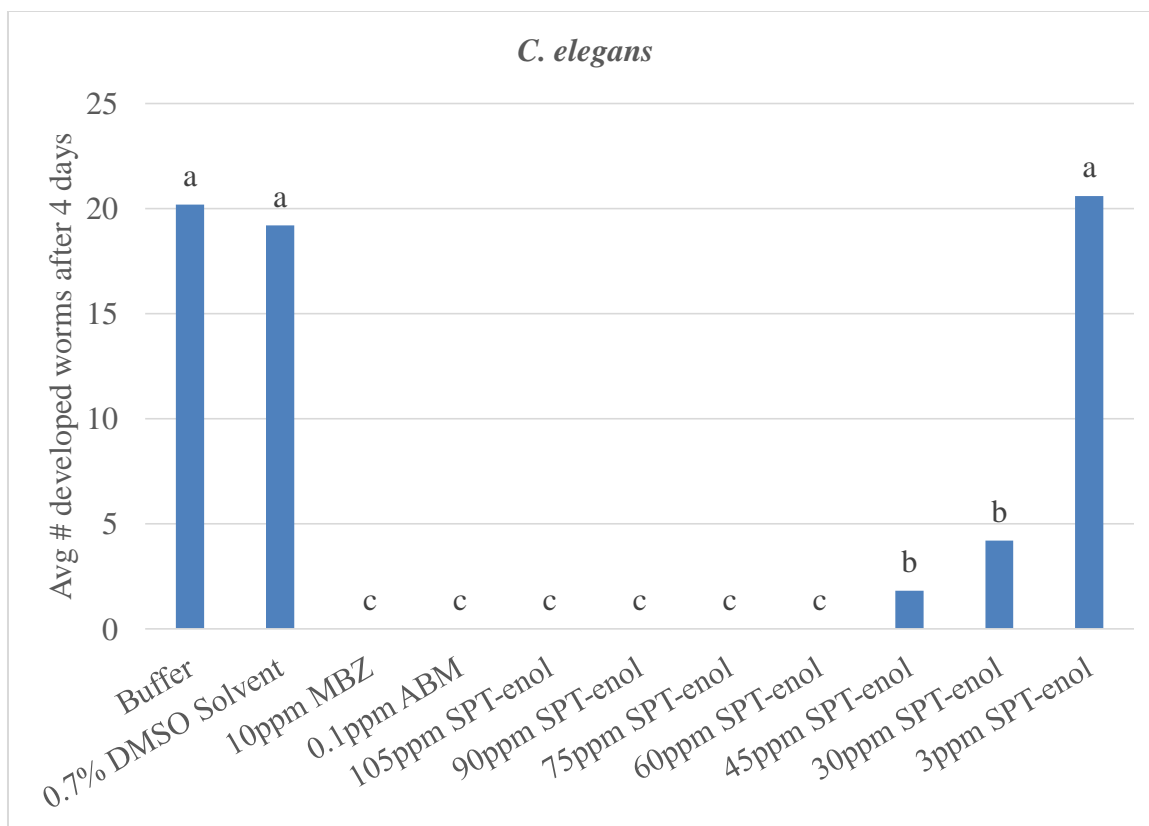


Figure 6. Number of *C. elegans* first stage juveniles that developed to adults in a range of spirotetramat-enol (SPT-enol) concentrations. A significant reduction in the number of worms that developed was observed in SPT-enol concentrations ≥ 30 ppm. Differing letters represent a statistical difference (Scheffé test, $p < 0.05$). Data were transformed ($\log(y+1)$) prior to statistical analysis; however, non-transformed data are presented in the graphs for ease of interpretation. Data are from a single representative staged assay (Experiment 1) with *C. elegans*.

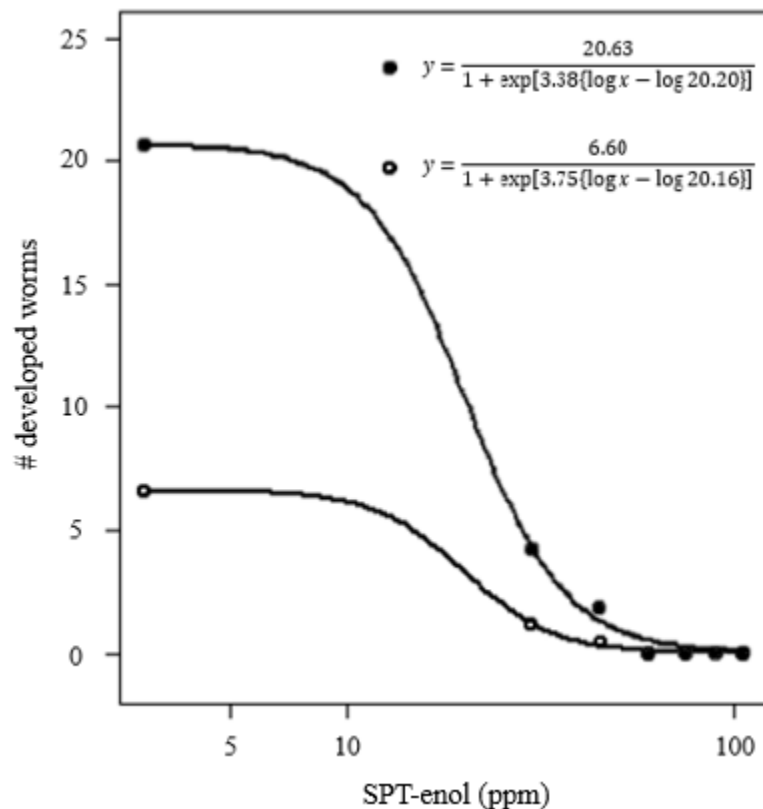


Figure 7. Log-logistic curves modeling the relationship between spirotetramat-enol (SPT-enol) concentration and development of *C. elegans* first-stage juveniles to adults generated from original data of two independent assays with staged *C. elegans*. Filled circles represent data from Experiment 1; open circles represent data from Experiment 2. The EC₉₅ is estimated to be 48ppm for Experiment 1 and 44ppm for Experiment 2.

**OPTIMIZATION OF TIMING FOR FOLIAR APPLICATION OF
SPIROTETRAMAT FOR MANAGEMENT OF PARASITIC NEMATODES
INFECTING PLANT ROOTS**

ABSTRACT

Spirotetramat (MoventoTM), a lipid-biosynthesis inhibiting insecticidal compound manufactured by Bayer CropScience, has also demonstrated potential as a nematicide. This compound is also unique in having basipetal and acripetal systemic movement in plants. The objective of this research was to evaluate the optimal time to spray spirotetramat (SPT) on plant foliage for systemic effects on nematodes infecting the plant's roots. SPT (Movento 240SC) was applied to plant foliage at 87.6 g ai/ha (5 fl oz/acre) in the greenhouse at 1-week intervals starting 1 week prior to inoculation with eggs of *Heterodera glycines* (soybean plants) or *Meloidogyne incognita* (tomato plants) and ending applications at 3 weeks after inoculation. SPT significantly inhibited nematode development to reproductive maturity when applied 1-2 weeks after inoculation. Optimal SPT application timings coincided with early stages of root infection, when nematodes are in vulnerable juvenile stages.

INTRODUCTION

Root-knot (*Meloidogyne* spp.) and soybean cyst (*Heterodera glycines*) nematodes are two of the most economically important plant-parasitic nematodes globally (Sasser and Freckman, 1987; Davis and Tylka, 2000; Mitkowski and Abawi, 2003). Both root-knot and soybean cyst nematodes are sedentary endoparasites that spend a majority of their parasitic cycles embedded within host roots. The diagnostic symptoms of root-knot nematodes are

intercalary galls or “knots” that form on host roots, while the diagnostic sign of soybean cyst nematodes is the presence of white to yellow swollen females (cysts) protruding from host roots. Above-ground symptoms for both nematodes are similar and include symptoms of nutrient deficiency and water stress, such as stunting, yellowing, and wilting, and, most importantly, decreased yield (Davis and Tylka, 2000; Karssen et al., 2013; Turner and Subbotin, 2013).

The range of host plant species for the major species of root-knot nematodes makes nematode management using crop rotations a challenge, whereas the limited host range of the soybean cyst nematode allows greater management efficacy in rotations to non-host crops like corn (Cook and Evans, 1987; Kerry, 1987; Viaene et al., 2013). Host resistance to root-knot and cyst nematodes can provide effective management when genetic sources of resistance can be incorporated into commercial cultivars, but the development of resistance-breaking nematode populations remains a constant threat (Starr et al., 2013).

Chemical control (soil fumigants and nonfumigant nematicides) can offer an effective means of management of plant-parasitic nematodes that is independent of the nematode species present, availability of host resistance or crop rotation strategies, and most environmental conditions. Chemical control of nematodes has been in practice since the late 1800s, often used in combination with other nematode management methods for maximum effectiveness. However, a major problem that has led to the decline of available nematicides in recent years is their inherent toxicity and potential to cause environmental damage. Since

the late 1970's bans and restrictions of a majority of nematicides has left a dire need for new chemical nematode management solutions (Hague and Gowen, 1987; Haydock et al., 2013).

Spirotetramat (MoventoTM) insecticide is a Bayer CropScience product that has also shown promising activity against nematode pests such as root-knot and soybean cyst nematodes. First shown to suppress nematode populations by McKenry et al. (2009), it has since been demonstrated that spirotetramat (SPT) may be acting by causing an arrest of nematode juvenile development to adulthood (Bayer CropScience, pers. comm.; results presented in Chapter 2, this thesis).

SPT can be applied in the field to plant foliage, where it then penetrates the leaf surface and is hydrolyzed *in planta* to the active enol form. The enol is capable of entering both phloem and xylem transport systems of the plant, resulting in unique two-way systemicity that allows the active ingredient to travel from the point of application to shoots and down into roots where nematodes and some insects feed (Fischer and Weiß, 2008; Vermeer and Baur, 2008).

While several studies have been conducted to compare different SPT application rates for nematode management in several crops (Bayer CropScience, pers. comm.), little has been done to determine optimal application timings. Several studies have shown that curative treatments tend to reduce nematode damage better than preventative treatments (Bayer CropScience, pers. comm.). No known studies have tested multiple application timings during the first life cycle of infecting nematodes to determine the best time to apply SPT to target susceptible nematode life stages for optimal nematode control. In field trials testing the

efficacy of SPT against *Heterodera avenae* infecting wheat, it was suggested that differences in the magnitude of reduction of *H. avenae* density in soil at different field locations could be due to different application timings with respect to the developmental stages of infecting nematodes (Smiley et al., 2011). Further research was suggested to determine optimal SPT application timings with respect to nematode developmental stages.

The objective of this study was to determine the optimal time to apply one application of SPT to plant foliage for maximum effects on either root-knot or soybean cyst nematodes infecting roots. This was accomplished by applying SPT at the labeled insecticidal rate of 87.6 g ai/ha (Movento 240SC @ 5 fl oz/acre) to greenhouse grown plants at 1-week intervals over the course of a single nematode life cycle. This would determine whether timing applications to coincide with vulnerable juvenile stages would result in increased efficacy.

MATERIALS AND METHODS

Nematode culture: *H. glycines* were propagated on roots of soybean plants (*Glycine max* cv Hutcheson) grown in the greenhouse. Cysts were dislodged from soybean roots with a directed water stream and collected in a 250 μ m sieve. Cysts were then crushed with a rubber stopper over a 25 μ m sieve to collect eggs (Goellner et al., 2001). *Meloidogyne incognita* were propagated on roots of tomatoes (*Solanum lycopersicum* cv Rutgers) grown in the greenhouse. *M. incognita* eggs were extracted from host roots by rinsing infected roots in 0.6% sodium hypochlorite for 30 seconds as previously described (Hussey and Barker, 1973) and then collecting and rinsing the eggs on a 25 μ m-opening sieve. Both *H. glycines* and *M. incognita* eggs were cleaned via the sugar flotation method for counting (Agrios, 2005).

Infection assays of H. glycines in SPT-treated soybean plants: Formulated SPT (Movento SC 240) was applied once at the labeled insecticidal rate of 87.6 g ai/ha (5-fl oz/acre) mixed in 10 gal of water, a rate equivalent to 936ppm, with a spray bottle to leaves of soybean plants (*Glycine max* cv Hutcheson) grown in pure sand in the greenhouse. A methylated seed oil (MSO) concentration adjuvant, consisting of methylated oils and a nonionic surfactant (NIS) blend (Bayer CropScience), was mixed into the spray bottle at a rate of 0.25% v/v. Plants were taken outside the greenhouse and sprayed to leaf drip. Plants were allowed to dry before being moved back into the greenhouse. Each assay consisted of seven treatments, with five plants per treatment positioned in a randomized complete block design. Treatments consisted of a non-treated control, an adjuvant control applied 1 week after inoculation, and Movento applied either 1 week before nematode inoculation (soybean seedlings at 3 weeks after germination), at the time of inoculation, or 1, 2, or 3 weeks after inoculation with 10,000 *H. glycines* eggs. Cysts were collected from each treated plant at 4 weeks after inoculation. After counting, cysts were crushed to release and count the eggs. Egg collections had relatively little soil debris, so cleaning via sugar flotation was not necessary for counting. Roots were also weighed at cyst harvest and cyst/g root, eggs/cyst, and egg/g root were calculated.

Infection assays of M. incognita in SPT-treated tomato plants: Formulated SPT (Movento SC 240) was applied once at the labeled insecticidal rate of 87.6 g ai/ha (5-fl oz/acre) to leaves of tomato plants (*Solanum lycopersicum* cv Rutgers) grown in pure sand in the greenhouse. Applications of SPT were made as previously described for soybeans. Each

assay consisted of seven treatments, with at least five plants per treatment arranged in a randomized complete block design. Treatments consisted of a non-treated control, an adjuvant control applied 1 week after inoculation, and SPT applied either 1 week before nematode inoculation (tomato plants at 5 weeks after germination), at the time of inoculation, or 1, 2, or 3 weeks after inoculation. Each plant was inoculated with 10,000 *M. incognita* eggs. Roots of each plant for the first assay were rinsed with water at 4 weeks after inoculation, weighed, and then stained with 0.03-g/L Phloxine B for counting egg masses (Fenner, 1962; Holbrook et al., 1983). Few egg masses were present on control roots after 4 weeks, so only galls were counted. The next two assays with *M. incognita* were taken down at 8 weeks after inoculation to allow more time for egg production. Stained egg masses were counted and then eggs were extracted from the roots for counting using 0.6% sodium hypochlorite as described above. After counting, egg masses/g root, eggs/egg mass, and eggs/g root were calculated.

Statistical analysis: Data was log transformed prior to analysis to satisfy assumptions of normality and homogeneity of variance ($\log(y)$ for both *H. glycines* assays and the first *M. incognita* assay with gall counts, and $\log(y+1)$ for the second and third *M. incognita* assays with egg mass and egg counts). A two-way ANOVA was performed for duplicate experiments using the general linear model (GLM) procedure in SAS version 9.4 (SAS Institute, Cary, NC) to check for experiment by treatment interactions. If interaction effects were not significant then data remained combined for subsequent analysis. If interaction effects were significant ($p < 0.05$) then further analysis was conducted on individual

experiments. When treatment means were significant, means were separated using the Scheffé test ($\alpha=0.05$).

RESULTS

Infection assays with H. glycines: Two-way ANOVA did not indicate any significant ($p<0.05$) experiment by treatment interactions, so the data from two independent assays were combined for further analysis. Development of *H. glycines* cysts per gram root at 4 weeks after inoculation was significantly reduced when SPT was applied at the time of inoculation, 1 week after inoculation, or 2 weeks after inoculation. Maximum reductions in cysts per gram root occurred when SPT was applied at 1 week after inoculation (Figure 8A). Reductions in the number of eggs per gram root followed the same pattern as cysts per gram root (Figure 8B) with regard to timing of SPT application. However, when the number of eggs per cyst was calculated, the only SPT treatment that provided significant reductions was SPT application at 2 weeks after inoculation (Figure 8C).

Infection assays with M. incognita: Two-way ANOVA indicated significant ($p<0.05$) experiment by treatment interactions, so further analyses were conducted on each independent assay. Representative results from the first assay are presented in Figure 9. Reductions in egg masses per gram root and eggs per gram root at 8 weeks after inoculation were significant for both assays when SPT was applied at 1 or 2 weeks after inoculation. Results were inconsistent across both assays for other SPT application timings. No significant reductions in eggs per egg mass were observed for either assay, although there was a numerical trend for increasing reductions in eggs per egg mass from SPT applications

made from the time of inoculation to 3 weeks after inoculation. In one additional assay, galls per gram root were counted at 4 weeks after inoculation, with no significant reductions observed for any SPT application timing as shown in Figure 10.

DISCUSSION

Nematode development to reproductive maturity was consistently inhibited in the plant-parasitic nematode species *H. glycines* and *M. incognita* when spirotetramat (SPT) was applied one time to host plant foliage at the labeled insecticidal rate of Movento at either 1 or 2 weeks after root inoculation with nematodes. Development of *H. glycines* cysts as well as production of eggs per gram of root were significantly reduced compared to non-treated controls when SPT was applied to soybean plants 1 or 2 weeks after inoculation. Production of *M. incognita* egg masses and eggs per gram of root were also significantly reduced compared to non-treated controls when SPT was applied to tomato plants either 1 or 2 weeks after inoculation, while reductions were inconsistent across assays for other application timings.

Optimal application timings of 1 or 2 weeks after inoculation coincide with early stages of root infection when nematodes are in the juvenile stages. At the time of inoculation, most nematodes would likely still be inside the eggs, although some would hatch immediately to J2s that are able to penetrate plant roots. Juveniles of endoparasitic plant nematodes like *H. glycines* and *M. incognita* would most likely be in the parasitic J2-J3 stages within roots at one week after inoculation. By 2 weeks after inoculation, most *H. glycines* and *M. incognita* within host roots would be in the J3-J4 stages; although, root-knot

nematode feeding is paused during these stages and resumes upon completion of the 4th molt (Karssen et al., 2013). Assays with *C. elegans* revealed significantly lower numbers of juveniles fully developed in SPT-enol concentrations ≥ 30 ppm, indicating an arrest of juvenile development (results presented in Chapter 2, this thesis). In the present study, similar results were observed. When SPT was applied at times coinciding with early juvenile development, significant reductions in adult females (cysts) were observed in *H. glycines*. This supports the hypothesis that SPT interferes with development of nematode juveniles into adults. When SPT was applied 3 weeks after inoculation many nematodes were developing to reproductive maturity - this was further confirmed since no significant cyst reductions were observed with SPT treatment at 3 weeks after inoculation. A slightly slower developmental rate of *M. incognita* to reproductive maturity (adult female) may explain the significant decrease in egg production with SPT treatment at 3 weeks after inoculation (although this specific observation was inconsistent among repeat experiments).

SPT applications made at 1 week prior to inoculation and at the time of inoculation did not provide consistent statistically significant reductions in *H. glycines* cyst or *M. incognita* egg mass counts. Results are consistent with previous research which indicated curative application timings are more effective than preventative application timings (Bayer CropScience, pers. comm.) The EC₉₅ for SPT-enol on *C. elegans* is approximately 44-48ppm (results presented in Chapter 2, this thesis). The lack of efficacy for preventative application timings suggests that SPT-enol concentrations in plant roots had dropped below the effective concentration by the time nematodes began to feed just a few days later, indicating a short

residual effect of less than one week. Spirotetramat is known to be particularly effective against a variety of sucking insect pests, so ingesting the SPT-enol form at effective concentrations from host plants is likely key to activity against target nematodes and insects. Symptoms in aphids feeding from SPT-treated plants included incomplete molting and subsequent death (Nauen et al., 2008). In a field setting, repeat applications of SPT may be needed to combat a potential short residual of effective SPT-enol concentrations in plants, although correct timing of a single application as demonstrated here can provide significant reduction in nematode development to reproductive maturity.

The lack of reduction in galls per gram root for *M. incognita*-infected tomatoes suggests that SPT does not prevent nematodes from invading roots, regardless of application timing. It is known that insects must ingest SPT for observable effects (Nauen et al., 2008). The data presented here also suggest that nematodes must infect and feed from roots in order to be affected by SPT. Once *M. incognita* invade roots, the symptom of gall formation occurs within 24-48 hours (Perry et al., 2013). Thus gall formation likely occurs before nematodes have had adequate time to feed and be exposed to SPT to the degree that effects on development and gall formation would be observed. However, once the nematodes have been exposed to adequate levels of SPT through feeding activity, juvenile development could be inhibited and reduced development of mature females and egg production would then be observed, even on heavily galled roots. Consequently, using a galling index is not recommended for rating the efficacy of SPT applications. Experiments to expose (non-feeding) hatched J2 to SPT-enol in solution or soil application prior to root infection may be

able to demonstrate whether nematode ingestion of SPT-enol is critical to its effects on subsequent nematode development.

The observed reductions in eggs per gram root were likely due to reduced total numbers of juveniles that developed to become gravid females. There was limited evidence for a reduction in fecundity with *H. glycines* when SPT was applied 2 weeks after inoculation, but no significant reduction in *H. glycines* fecundity observed at 1 week or 3 weeks after SPT application. Numerical reductions in the number of eggs plus juveniles per cyst were also observed in *H. avenae* in wheat, but reductions in fecundity were not statistically different from the non-treated controls (Smiley et al., 2011). The lack of any significant reduction in eggs produced per *M. incognita* female in the study here at any SPT treatment time point suggests limited effects of SPT on nematode fecundity.

The present study showed that SPT consistently reduces *H. glycines* and *M. incognita* development to reproductive maturity with a single application to foliage at 1-2 weeks after inoculation with nematodes. If these timings were to be translated into a field setting, SPT applications should be made early in the growing season, when the first generation of nematodes is just starting to infect and develop in roots. However, since SPT must be absorbed by plant foliage in order to be translocated to the roots where nematodes feed, applications should not be made before sufficient foliage is present to absorb the active ingredient. Further studies could be done in a field setting to balance the need for early applications targeting nematodes in juvenile stages and requirements of sufficient plant foliage to receive application. Combining use of SPT with other early season chemical or

biological nematode control agents targeted at reducing the incidence and/or severity of nematode invasion of roots should also be further investigated.

The labeled rate of SPT (for insect control) applied to plants in this study was equivalent to 936ppm of active ingredient; however, assays with *C. elegans* have indicated that EC₉₅ of SPT-enol for inhibition of juvenile development is between 44 and 48ppm (results presented in Chapter 2, this thesis), approximately one twentieth of the applied rate. It is unknown how much of the applied SPT is absorbed by plant foliage, converted into the active enol form, and translocated down to roots where nematodes feed. However, our data suggest that within one week after Movento application at the labeled rate, SPT-enol levels drop below the effective concentration to significantly inhibit nematode development in roots. Further research is suggested to determine the percentage and titer of foliar-applied SPT that becomes available to nematodes in plant roots and how quickly the available SPT-enol can be metabolized by the plant. Such information would likely allow field performance data to be correlated with data obtained from lab assays. Application rates could then be adjusted accordingly to ensure adequate concentrations/residual of SPT-enol in roots (at least 44ppm), without applying excess formulated product to plant foliage. Knowing how much SPT-enol initially reaches the roots and how long it stays there could help inform decisions of whether a second application is needed. Perhaps higher rates of SPT could be applied to increase the titer/residual of SPT-enol in the roots for longer-lasting effects or second applications could be made to target subsequent nematode generations. Fine-tuning

application rates in this way could allow more efficient use of SPT, reducing management costs without compromising efficacy.

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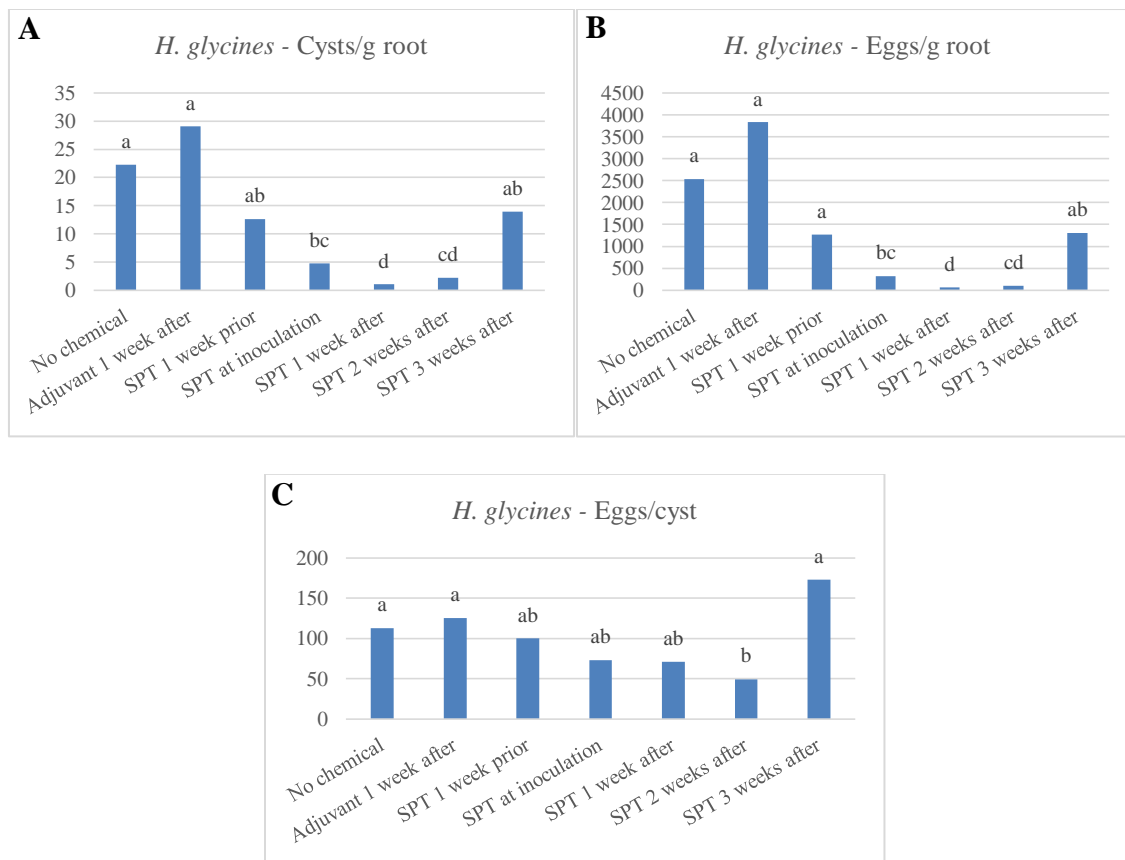


Figure 8. Reproduction of *Heterodera glycines* in roots of soybean plants upon single foliar treatment with spirotetramat (Movento) at different time intervals. Development of *H. glycines* cysts per gram root (A) and eggs per gram root (B) were maximally reduced at 4 weeks after inoculation when SPT was applied 1 or 2 weeks after inoculation, although, significant reductions were also observed when SPT was applied at inoculation. Significant reductions in eggs per cysts were only observed when SPT was applied 2 weeks after inoculation (C). Differing letters represent a statistical difference (Scheffé test, $p < 0.05$). Data were transformed prior to statistical analysis ($\log(y)$); however, non-transformed data are presented in the graphs for ease of interpretation. The data presented represent an average across two independent assays.

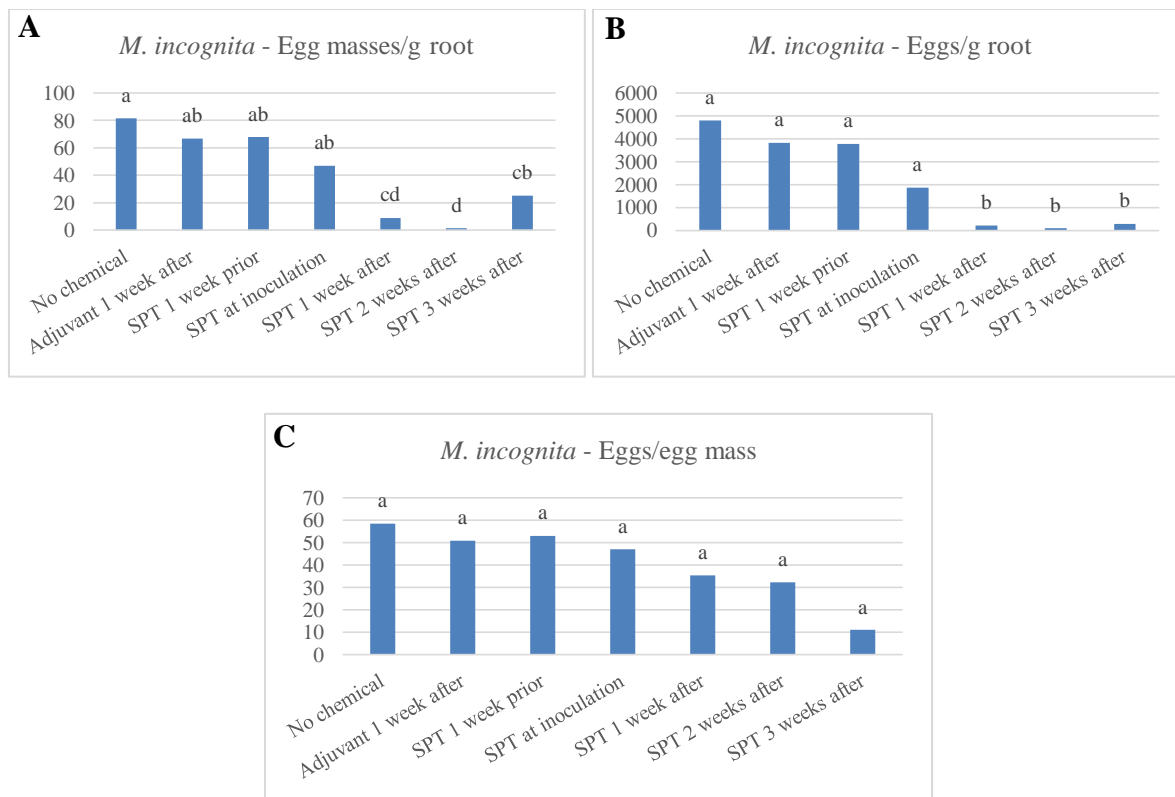


Figure 9. Reproduction of *Meloidogyne incognita* in roots of tomato plants upon single foliar treatment with spirotetramat (Movento) at different time intervals. Production of egg masses per gram root (A) was significantly reduced at 8 weeks after inoculation when SPT was applied 1 or 2 weeks after inoculation. Total egg numbers per gram root (B) were also significantly reduced when SPT was applied 1 or 2 weeks after inoculation, as well as when SPT was applied 3 weeks after inoculation. No significant reductions in eggs per egg mass were observed for any application timing of SPT (C). Differing letters represent a statistical difference (Scheffé test, $p < 0.05$). Data were transformed prior to statistical analysis ($\log(y+1)$); however, non-transformed data are presented in the graphs for ease of interpretation. The data presented are from one representative assay with *M. incognita* since two-way ANOVA indicated significant ($p < 0.05$) experiment by treatment interactions.

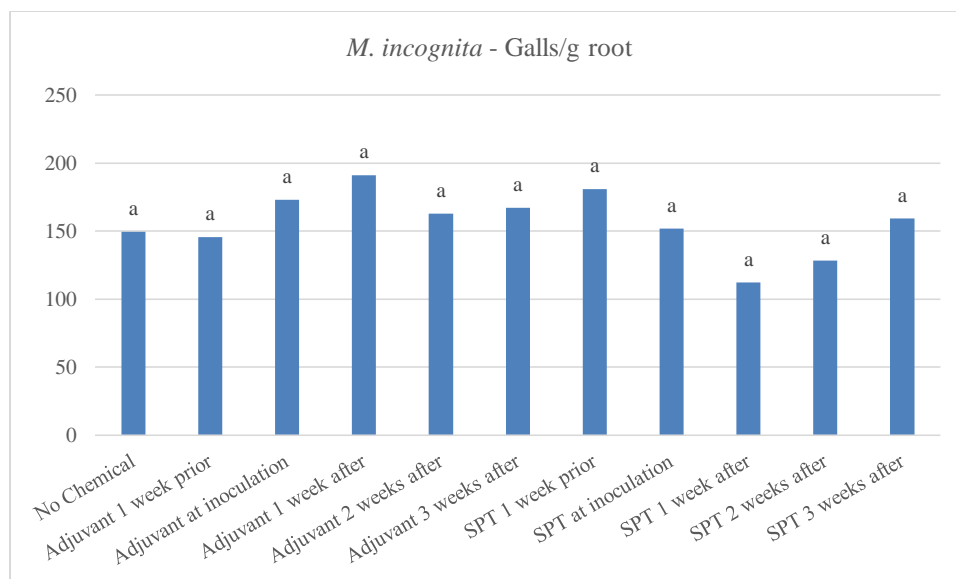


Figure 10. Galls induced by *Meloidogyne incognita* in roots of tomato plants upon single foliar treatment with spirotetramat (Movento) at different time intervals. No significant reductions in root galling by *M. incognita* at 4 weeks after inoculation were observed for any application timing of SPT. Differing letters represent a statistical difference (Scheffé test, $p < 0.05$). Data was transformed prior to statistical analysis ($\log(y)$); however, non-transformed data is presented in the graphs for ease of interpretation. The data presented is from one representative assay with *M. incognita*.